Translational Oncogenomics and Human Cancer Interactome Networks: Techniques and Complex System Dynamic Approaches

Review

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Abstract

An overview of translational, human oncogenomics, transcriptomics and cancer interactomic networks is presented together with basic concepts and potential, new applications to Oncology and Integrative Cancer Biology. Novel translational oncogenomics research is rapidly expanding through the application of advanced technology, research findings and computational tools/models to both pharmaceutical and clinical problems. A self-contained presentation is adopted that covers both fundamental concepts and the most recent biomedical, as well as clinical, applications. Sample analyses in recent clinical studies have shown that gene expression data can be employed to distinguish between tumor types as well as to predict outcomes. Potentially important applications of such results are *individualized* human cancer therapies or, in general, 'personalized medicine'. Several cancer detection techniques are currently under development both in the direction of improved detection sensitivity and increased time resolution of cellular events, with the limits of single molecule detection and picosecond time resolution already reached. The urgency for the complete mapping of a human cancer interactome with the help of such novel, high-efficiency / low-cost and ultra-sensitive techniques is also pointed out.

Key Words:

Translational Oncogenomics and Integrative Cancer Biology in clinical applications and individualized cancer therapy/Pharmacogenomics; cancer clinical trials with signal pathways inhibitors; high-sensitivity and high-speed microarray techniques (cDNA, oligonucleotide microarrays, protein arrays and tissue arrays) combined with novel dynamic NIR/fluorescence cross-correlation spectroscopy and dynamic microarray techniques; recent human cancer interactome network models of high-connectivity cancer proteins; global topology and Complex System Dynamics of the human cancer Interactome and differential gene expression (DGE) in human lung cancer; epigenomics in mammalian cells and development of new medicines for cancer therapy.

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1. Introduction

1.1. Current Status in Translational Genomics and Interactome Networks

Upon completion of the maps for several genomes, including the human genome, there are several major post-genomic tasks lying ahead such as the translation of the mapped genomes and the correct interpretation of huge amounts of data that are being rapidly generated, or the important task of applying these fundamental results to derive major benefits in various medical and agricultural biotechnology areas. It follows from the 'central dogma' of molecular biology that *translational genomics* is at the center of these tasks that are running from *transcription* through *translation* to *proteomics* and *interactomics*. The *transcriptome* is defined as the set of all 'transcripts' or messenger RNA (mRNA) molecules produced through transcription from DNA sequences by a single cell or a cell population. This concept is also extended to a multi-cellular organism as the set of all its transcripts. The transcriptome thus reflects the active part of the genome at a given instant of time. *Transcriptomics* involves the determination of mRNAs expression level in a selected cell population. For example, an improved understanding of cell differentiation involves the determination of the stem cell transcriptome; understanding carcinogenesis requires the comparison between the transcriptomes of cancer cells and untransformed ('normal') cells. However, because the levels of mRNA are not directly proportional to the expression levels of the proteins they are encoding, the protein complement of a cell or a multi-cellular organism needs to be determined by other techniques, or combination of techniques; the complete protein complement of a cell or organism is defined as the *proteome*. When the network (or networks) of complex protein-protein interactions (PPIs) in a cell or organism is (are) reconstructed, the result is called an '*interactome*'. This complete network of PPIs is now thought to form the 'backbone' of the signaling pathways, metabolic pathways and cellular processes that are required for all key cell functions and, therefore, cell survival. Such a complete knowledge of cellular pathways and processes in the cell is essential for understanding how many diseases -- such as cancer (and also ageing) —originate and progress through mutation or alteration of individual pathway components. Furthermore, determining human cancer cell interactomes of therapy-resistant tumors will undoubtedly allow for rational clinical trials and save patients' lives through individualized cancer therapy.

Since the global gene expression studies of DeRisi et al. in 1997, translational genomics is very rapidly advancing through the detection in parallel of mRNA levels for large numbers of molecules, as well as through progress made with miniaturization and high density synthesis of nucleic acids on microarray solid supports. Gene expression studies with microarrays permit an integrated approach to biology in terms of network biodynamics, signaling pathways, protein-protein interactions, and ultimately, the cell interactome. An important emerging principle of gene expression is the *temporally coordinated regulation* of genes as an extremely efficient mechanism (Wen *et al* 1998) required for complex processes in which all the components of multi-subunit complexes must be present/available in defined ratios at the same time whenever such complexes are needed by the cell. The gene expression profile can be thought of either as a 'signature/ fingerprint' or as *a molecular definition of the cell in a specified state* (Young, 2000). Cellular phenotypes can then be inferred from such gene expression profiles. Success has been achieved in several projects that profile a large number of biological samples and then utilize pattern matching to predict the function of either new drug targets or previously uncharacterized genes; this '*compendium approach'* has been demonstrated in yeast (Gray *et al* 1998; Marton *et al*, 1999; Hughes *et al* 2000), and has also been applied in databases integrating gene expression data from pharmacologically characterized human cancer lines (NCI60, http://dtp.nci.nih.gov) ,

or to classify cell lines in relation to their tissue of origin and predict their drug resistance or chemosensitivity (Weinstein *et al*, 1997; Ross *et al* 2000, Staunton *et al* 2001). Furthermore, sample analyses in clinical studies have shown that gene expression data can be employed to distinguish between tumor types as well as to predict outcomes (Golub *et al* 1999; Bittner *et al*, 2000; Shipp et al 2002). The latter approach seems to lead to important applications such as individualized cancer therapy and 'personalised medicine'. On the other hand, such approaches are complemented by studies of protein-protein interactions in the area called *proteomics*, preferably under physiological conditions, or more generally still, in *cell interactomics.* Several technologies in this area are still developing both in the direction of improved detection sensitivity and time resolution of cellular events, with the limits of single molecule detection and picosecond time resolution already attained. In order to enable the development of new applications such techniques will be briefly described in the next section, together with relevant examples of their recent applications.

1.2. Basic Concepts in Transcription, Translation and Interactome Networks: The Analysis of Bionetwork Dynamics

     Protein synthesis as a channel of information operates through the formation of protein amino acid sequences of polypeptides via *translation* of the corresponding polynucleotide sequences of (usually single –stranded, **messenger**) ribonucleic acid, that is:

DNA (**gene**) *transcription* ➔ **mRNA--**➔*translation* **into** Amino-acid polypeptide sequence---➔**protein** (quaternary) *assembly* from polypeptide subunits.

   Although not shown in this scheme, several key enzymes make such processes both efficient and precise through
highly-selective catalysis; moreover, the protein assembly involves both specific enzymes and ribosome 'assembly lines'. Furthermore, such processes are compartmented in the mammalian cells by selective intracellular membranes; this seems to be also important for cell cycling and the control of cell division.

   On the other hand, the *reverse transcription, RNA-➔DNA,* does also occur (under certain conditions), catalized by a reverse transcriptase that contains both polypeptide chains and an RNA (master) strand.

   If error free, the first of these two sequence of processes —which are of fundamental biological importance-- generates true replicas of the information contained in the *sense codons* of the genes that are transcribed into mRNA *anti-codons*. (Recall also that DNA stores information in the neucleotide bases A (Adenine), C (Cytosin), G (Guanine) and T (Thymine), and that a triplet of such nucleotides in the DNA sequence is called a *codon,* which may encode unambiguously just the information necessary to specify a single amino acid. Moreover, the genetic code is a redundant one and without any overlap; the code is quasi-universal, and also capable of 'reverse transcription' from certain types of RNA back into DNA, as shown above in the second sequence of processes). Notably also, not all nucleotide or codon sequences present in the genome (DNA) are transcribed *in vivo*. Typically only a small percentage is transcribed. The transcribed (mRNA) sequences form what is naturally called the *transcriptome;* the protein--encoded version of the transcriptome is called the *proteome*, and upon including all protein--protein interactions for various cellular states one obtains the (global) *interactome* network. More

generally, biological interactive networks as a class of complex bionetworks consist of local cellular communities (or *'organismic sets'*) that are organized and managed by their characteristic selection procedures. Thus, in any partitioning of the organismal, or cell, structure, it is often necessary to regulate the *local* properties of the organism rather than the *global* mechanism, which explains an organism's need for specialized, 'modular constructions'. Such a modular, complex system biology approach to modeling signaling pathways and modifications of cell-cycling regulatory mechanisms in cancer cells was recently reported (Baianu, 2004); several consequences of this approach were also considered for the proteome and interactome networks in a 'prototype' cancer cell model (Prisecaru and Baianu, 2005).

Note, on the other hand, that there seem to be also present in the living cell certain proteins and enzymes that are involved in *global* intra-cellular interactions which are thought to be essential to the cell survival and cell's flexible adaptation to stresses or challenge.

Let us consider first the well-known example of *gene clustering* in microbial organisms. Jacob and Monod (1961a,b) have shown, that in the bacterium *Escheria Coli* a "*regulatory gene*" and three "structural genes" concerned with lactose metabolism lie near one another in the same region of the chromosome. Another special region near one of the structural genes has the capacity of responding to the regulatory gene, and it is called the "operator gene". The three structural genes are under the control of the same operator and the entire aggregate of genes represents a functional unit or "*operon*". The presence of this "clustering" of genes seems to be doubtful in the case of higher organisms although in certain eukaryotes, such as yeast (*Saccharomyces cerevisiae*), there is also evidence of such gene clustering; this has important consequences for the dynamic structure of the cell interactome which is thought to be neither random nor linear, although the experimental evidence so far is neither extensive nor generally accepted.

It would seem, therefore, natural to define any assembly, or aggregate, of interacting genes—*even in the absence of local gene clustering* -- as a '*genetic network*' (that is, without considering the 'clustering' of genes as a necessary, or essential, condition for the existence of such bionetworks in all biological organisms). Genetic information thus affords a hierarchical structure within which genetic switches operate as transcription factors that are switching on other genes within this hierarchy. More specifically, the functions of inter--regulatory systems of genetic networks *via* activation or inhibition of DNA transcription can be understood in terms of models at several differing levels where various factors influence distinct states usually by some embryonic process, or by the actual network structure itself. Moreover, the regulation of genetic information transfer can occur either at the level of transcription or at the level of translation. *Epigenetic* controls may, in addition, play key roles in developmental processes and neoplastic transformations through the (bio) chemical modification of gene structure and expression under physiological conditions.

For each gene network it is important to understand the dynamics of inter--regulatory genetic groups which of themselves create hierarchical systems with their own characteristics. A gene positively (or negatively) regulates another when the protein coding of the former activates (respectively, inhibits) the properties of the latter. In this way, genetic networks are comprised of inter--connecting positive and negative feedback loops. The DNA binding protein is encoded by a gene at a network vertex i say, activating a target gene j where the transcription rate of i is realized in terms of a function of the concentration $[x_j]$ of the regulatory protein. Acting towards a given gene, the regulatory genes are protein coded and induce a transcription factor. Recent

modeling techniques draw from a variety of mathematical sources, such as: topology (including graph theory), biostatistics, stochastic differential equations, Boolean networks, and qualitative system dynamics (Baianu, 1971a; de Jong *et al* 2000; 2003, 2004). Non--boolean network models of genetic networks and the interactome were also developed and compared with the results of Boolean ones (Baianu, 1977, 1984, 1987; Georgescu, 2006; Baianu, 2005; Baianu *et al. 2006).* The traditional use of comparatively rigid Boolean networks (reviewed extensively, for example in Baianu, 1987) can be thus extended through flexible, multi--valued (non--Boolean) logic algebra bionetworks with complex, *non-linear dynamic* behaviors that mimic complex systems biology (Rosen, 2000). The results obtained with such non--random genetic network models have several important consequences for understanding the operation of cellular networks and the formation, transformation and growth of neoplastic network structures. Non--boolean models can also be extended to include *epigenetic* controls, as well as to mimic the coupling of the genome to the rest of the cell through specific signaling pathways that are involved in the modulation of both translation and transcription control processes. The latter may also provide novel approaches to cancer studies and, indeed, to developing 'individualized' cancer therapy strategies and novel anti-cancer medicines targeted at specific signaling pathways involved in malignant tumors resistance to other therapies.

2.   Techniques and Application Examples

2.1. DNA Microarrays

DNA microarray technology is widely employed to monitor in a single experiment the gene expression levels of all genes of a cell or an organism. This includes the identification of genes that are expressed in different cell types as well as the changes in gene expression levels caused, for example, by differentiation or disease. The terabytes of data thus obtained can provide valuable clues about the interactions among genes and also about the interaction networks of gene products. It has been reported that cDNA arrays were pioneered by the Brown Laboratory at Stanford University (Brown and Botstein, 1999; URL: http://cmgm.stanford.edu/pbrown/mguide/index.html). Several quantitative and high-density DNA array applications were then reported in rapid succession (Schena et al 1995; Chee et al 1996; Brown and Botstein, 1999). Such microarrays are generated by automatically printing double-stranded cDNA onto a solid support that may be either glass silicon or nylon. The essential technologies involved are robotics and devlopment/selection of sequence-verified and array-formatted cDNA clones. The latter ensures that both the location and the identity of each cDNA on the array is known. Sequence-verified and array-formatted cDNA clone sets are now available from companies such as Incyte Genomics (Palo Alto, CA; URL: http://www.synteni.com/) and Research Genetics (Huntsville, AL; URL: http://www.resgen.com/). In cDNA-based gene expression profiling experiments, the total RNA is extracted from the selected experimental samples and the RNA is fluorescently labeled with either cye3- or cye5-dUTP in a single round of reverse transcription. The latter have several advantages: they are readily incorporated into cDNA by reverse transcription, they exhibit widely separated excitation and emission spectra, and also they possess good photostability. Such fluorescently--labeled cDNA probes are then hybridized to a single array through a competitive hybridization reaction. Detection of hybridized probes is achieved by laser excitation of the individual fluorescent markers, followed by scanning using a confocal scanning laser microscope. The raw data obtained with a laser scanning systems is represented as a normalized ratio of cye3: cye5 and automatically color coded; thus, red color is conventionally selected to represent those genes that are transcriptionally upregulated in the

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test versus the reference, whereas green color represents genes that are downregulated; those genes that exhibit no difference between test and reference samples are shown in yellow. The analysis of the gene expression data obtained by such a high throughput microarray technology is quite complex and requires advanced computational/bioinformatics tools as already discussed in Section 1.2. Other aspects related to interactomics will be discussed in Section 3. An alternative technology to cDNA microarrays will be discussed in the next section.

2.2. Oligonucleotide Arrays

By combining oligonucleotide synthesis with photolithography it was possible to synthesize specific oligonucleotides with a selected orientation onto the solid surface of glass or silicon chips (Lockhart *et al* 1996; Wodicka L, *et al* 1997), thus forming oligonucleotides arrays. The expression monitoring was then carried out by hybridization to high-density oligonucleotide arrays (Lockhart *et al* 1996; Wodicka L, *et al* 1997). Commercially available oligonucleotides array products from Affymetrix (Santa Clara, CA; http://www.affymetrix.com/) include human, mouse and several other organisms. Each gene included on the oligonucleotides array is represented by up to 20 different oligonucleotides that span the entire length of the coding region of that gene. To reduce substantially the rate of false positives, each of these oligonucleotides is paired with a second mismatch oligonucleotide in which the central base in the sequence has been replaced by a different base. As in the cDNA approach, fluorescently labeled probes are generated from test and reference samples in order to carry out comparative gene expression profiling. After cDNA amplification, the differential fluorescent signal is detected with a laser scanning system and provides a map of the alterations in the transcriptional profile between the test and reference samples that are being compared. Dynamic analysis and further sophistication is added to such oligonucleotides array capabilities by the techniques briefly discussed in Section 2.7.

The molecular classification of cancers is of immediate importance to both cancer diagnosis and therapy. Tumors with similar histologic appearance quite often have markedly different clinical response to therapy. Such variability is a reflection of the underlying cell line and molecular heterogeneity of almost any tumor. Gene expression profiling has been successfully employed for molecular classification of cancers. It would seem from available data that each patient has his/her own molecular identity signature or fingerprint (Mohr et al 2002). Thus, Ross *et al*. (2000) reported the gene expression analysis in 60 cancer cell lines utilized in the Developmental Therapeutics Program by the National Cancer Institute (NCI) at NIH (Bethesda, MD, USA); they reported that cell lines could be grouped together according with the organ type and specific expression profiles corresponded to *clusters of genes*. Similar findings were reported for ovarian and breast cancers; in the latter case, Perou *et al*. (2000) reported that specific epithelial cell line genes clustered together and are relevant in breast cancer subdivision into the basal- like and luminal groups. On the other hand, the eventual use of microarray technologies for clinical applications will involve the utilization of proteome and tissue arrays in addition to gene expression profiling by cDNA microarrays and oligonucleotides arrays. Thus, tissue markers revealed unexpected relationships, as in the case of gene expression analysis of small-cell lung carcinoma, pulmonary carcinoid tissue and bronchial epithelial tissue culture (Anbazhagan *et al* 1999). Because a single biomarker has serious limitations for clinical applications there is a need for a battery of disease biomarkers that would provide a much more accurate classification of cancers. High-density screening with microarray technologies is therefore valuable in

pharmacogenomic (individualized therapy), toxicogenomic, as well as in clinical--diagnostic investigations.

2.4. Proteome Arrays

In a manner similar to the transcriptome, the proteome does undergo both qualitative and quantitative changes during pathogenesis, and this is also true in carcinogenesis. Proteome array-based methodologies involve either proteins or protein-binding particles (DNA, RNAs, antibody, or other ligands). Utilizing such proteome arrays one can respectively study either differential protein expression profiling or protein-ligand interaction screening under specified, or selected, physiopathological conditions. According to Kodadek (2001), these two classes of practical applications of proteome arrays are respectively defined as *protein function* and *protein-detecting* arrays. A protein-detecting array may consist of an arrayed set of protein ligands that are employed to profile gene expression and therefore make visible *'proteosignatures'* characterizing a selected cellular state or phase. In view of the potential clinical importance of a proteomic survey of cancers, the 'hunt' is now on for such proteosignatures of cancer cells but the amount of data reported to date is still quite limited. Already, the coupling of proteome arrays with high-resolution chromatography techniques followed by mass spectrometry has provided powerful analytical tools with which one can profile the protein expression in cancer cells. For example, a ProteinChip^{TM} (Ciphergen Inc, Fremont, CA, USA) was successfully utilized to investigate the proteome of prostate, ovarian, head and neck cancer cells (von Eggeling et al 2000). Such methods identified *protein fingerprints* from which *cancer biomarkers* can also be obtained. A reverse proteome array was also reported in which many extracted proteins from a patient sample are 'printed' onto a flat, solid support (Paweletz *et al* 2001); this reverse system was then utilized to carry out a biochemical screening investigation of the signaling pathways in prostate cancer. Through such investigations it was found that the carcinoma progression was positively correlated with the phosphorylation state of Akt and negatively correlated with ERK pathways; furthermore, the carcinoma progression was positively correlated with the suppression of the apoptotic pathways, a finding which is consistent with the more detailed, recent reports on cyclin CDK2 and transcriptional factors affected by CDK2 that will be discussed in Section 4. Immunophentotyping of leukemias with antibody microarrays was also reported (Belov, de la Vega, dos Remedios, et al 2001), and does provide an increased antigen differentiation (CD) in leukemia processing.

2.5. Tissue Arrays

The logical step after the identification of potential cancer markers through genomic and/or proteomic array analysis is the evaluation of such cancer markers by tissue arrays/ tissue chips for diagnostic, prognostic, toxicogenomic and therapeutic relevance. Such tissue microarrays (TMAs) were often designed to contain up to 1000 sections of 5micron thick sections, usually chemically--fixed and arrayed upon a glass slide. TMAs allow large-scale screening of tissue specimens and can be utilized, for example, for the pathological evaluation of molecular irreversible changes that are important for cancer research and treatment. Therefore, they can speed up the process of translating experimental, or fundamental, discoveries into clinical practice and improved cancer treatments.

In conjunction with *fluorescence in situ hybridization* (FISH), TMAs have been utilized in cancer research to analyze in parallel the gene amplification in multiple tissue sections thus allowing the researchers to map the distribution of gene amplification throughout an entire tumor.

This also allowed the monitoring of changes in gene amplification during the cancer progression (Bubendorf et al 1999). Furthermore, utilizing immunohistochemical staining of tissue arrays it was possible to measure the protein levels in tumor specimens. Thus, topoisomerase II alpha was reported to be highly expressed in patients with the poorest prognosis in oligodendrogliomas (Miettinen et al 2000). TMAs may become a clinical validation, as well as a 'global' tool; thus, recent studies reported this technique to be highly efficient for the identification of molecular (irreversible) alterations during cancer initiation and progression (Lassus et al 2001).

A pathologist might, however, object that the tissue microarray provides only a partial analysis of the tumor. The data reported so far seems to indicate that with carefully designed sampling this may not be a serious problem; however, in view of the importance of the problem for clinical applications it should be systematically investigated as a function of sampling whenever this is feasible.

The array-based technologies briefly described above provide powerful means for functional analyses of cancer and other complex diseases. Undoubtedly, much more can, and will be, done with proteome or tissue arrays combined with other state-of-the-science spectroscopic techniques as suggested in the following sections 2.6, 2.7, 4 and 6.2. Especially, the following three sections 2.6 and 2.7 will illustrate how advanced, ultra-fast and super-sensitive techniques can be used in conjunction with either nucleic acids or proteome arrays to both speed up thousand-fold the microarray data collection (for nucleic acids, proteins, ligand-binding, etc.) and also increase sensitivity to its possible limit of single molecule detection.

2.6. Fluorescence Correlation Spectroscopy and Fluorescence Cross--Correlation Spectroscopy: Applications to DNA Hybridization, PCR and DNA Binding

In the bioanalytical and biochemical sciences Fluorescence Correlation Spectroscopy (FCS) techniques can be utilized to determine various thermodynamic and kinetic properties, such as association and dissociation constants of intermolecular reactions in solution (Thompson, 1991; Schwille, Bieschke and Oehlenschläger, 1997). Examples of this are specific hybridization and renaturation processes between complementary DNA or RNA strands, as well as antigene-antibody or receptor-ligand recognition. Although of significant functional relevance in biochemical systems, the hybridization mechanism of short oligonucleotide DNA primers to a native RNA target sequence could not be investigated in detail prior to the FCS/FCCS application to these problems. Most published models agree that the process can be divided into two steps: a reversible first initiating step, where few base pairs are formed, and a second irreversible phase described as a rapid zippering of the entire sequence. By competing with the internal binding mechanisms of the target molecule such as secondary structure formation, the rate-determining initial step is of crucial relevance for the entire binding process. Increased accessibility of binding sites, attributable to single-stranded open regions of the RNA structure at loops and bulges, can be quantified using kinetic measurements (Schwille, Oehlenschläger and Walter, 1996).

The measurement principle for nearly all FCS/FCCS applications is based so far upon the change in diffusion characteristics when a small labeled reaction partner (eg, a short nucleic acid probe) associates with a larger, unlabeled one (target DNA/RNA). The average diffusion time of the labeled molecules through the illuminated focal volume element is inversely related to the diffusion coefficient, and increases during the association process. By calibrating the diffusion characteristics of free and bound fluorescent partner, the binding fraction can be easily evaluated from the correlation curve for any time of the reaction. This principle has been employed to

investigate and compare the hybridization efficiency of six labeled DNA oligonucleotides with different binding sites to an RNA target in a native secondary structure (Schwille, Oehlenschläger and Walter, 1996). Hybridization kinetics was examined by binding six fluorescently labeled oligonucleotide probes of different sequence, length and binding sites to a 101-nucleotide-long native RNA target sequence with a known secondary structure (Fig.1). The hybridization kinetics was monitored and quantified by FCS, in order to investigate the overall reaction mechanism. In this "all-or-none" binding model, the expected second-order reaction was assumed to be irreversible. For nM concentrations and at temperatures around 40°C, the typical half-value reaction times for these systems are in the range of 30 to 60 min, and therefore the hybridization process could be easily followed by FCS diffusional analysis. At the measurement temperature of 40°C the probes are mostly denatured, whereas the target retains its native structure. The binding process could be directly monitored through diffusional FCS analysis, via the change in translational diffusion time of the labeled 17-mer to 37-mer oligonucleotide probes HS1 to HS6 upon specific hybridization with the larger RNA target (Figure 1 and Figure 2).
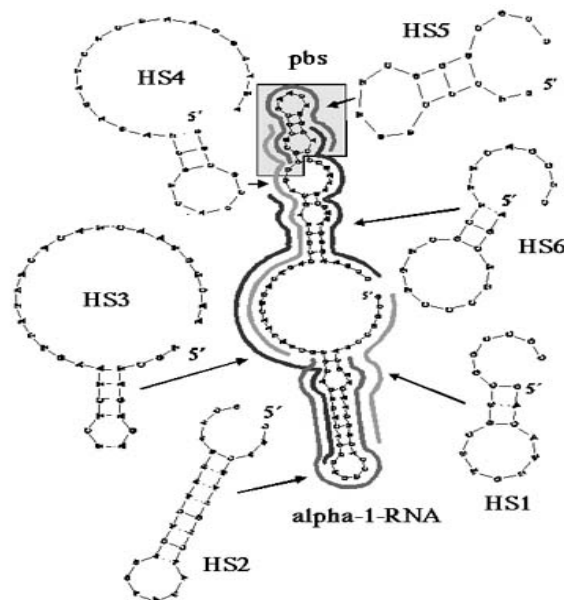
Fig. 1. Secondary structures and binding sites of the oligonucleotides HS1 to HS6 and the target RNA.

FCCS Applications to DNA Hybridization, PCR and DNA Binding

DNA hybridization & cleavage

*EcoR*I

R

C
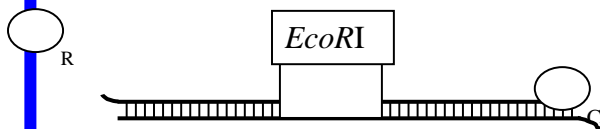
Kettling *et al.* **(1998)** *PNAS:* **95, 1416;**
Koltermann *et al.* **(1998)** *PNAS* **95, 1421;**
Schwille *et al.* **(1997)** *Biophys. J.* **:72, 1878 ;**
Winkler *et al.* **(1999)** *PNAS* **: 96, 1375.**

DNA polymerization chain reaction

Aut

C
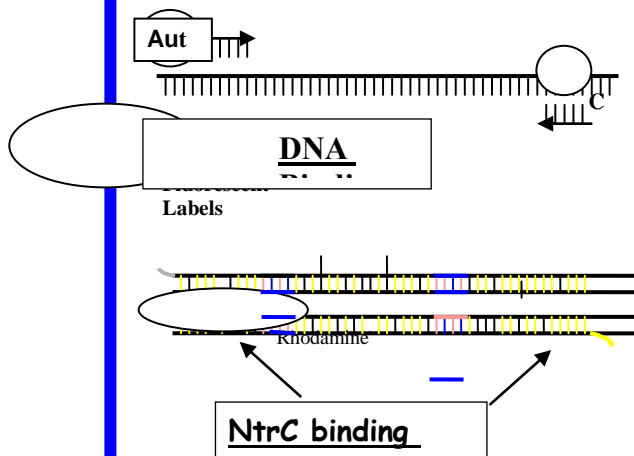
Rigler *et al.* **(1999)** *J. Biotechnology.*

DNA Binding

Labels

Rippe (2000) *Biochemistry.* **39:**

Rhodamine

NtrC binding

The characteristic diffusion time through the laser-illuminated focal spot of the 0.5 μm-diameter objective increased from 0.13 to 0.20 ms for the free probe, and from 0.37 to 0.50 ms for the bound probe within 60 min. The increase in diffusion time from measurement to measurement over the 60 min could be followed on a PC monitor and varied strongly from probe to probe. HS6 showed the fastest association, while the reaction of HS2 could not be detected at all for the first 60 min. It has been shown above that FCS diffusional analysis provides an easy and comparably fast determination of the hybridization time course of reactions between complementary DNA/RNA strands in the concentration range from 10^{-10} to 10^{-8} M. Perturbation of the system is therefore not necessary, so the measurement can be carried out at thermal equilibrium. Thus, the FCS-based methodology also permits rapid screening for suitable anti-sense nucleic acids directed against important targets like HIV-1 RNA with low consumption of probes and target.

Because of the high sensitivity of FCS detection, the same principle can be exploited to simplify the diagnostics for extremely low concentrations of infectious agents like bacterial or viral DNA/RNA. By combining confocal FCS with biochemical amplification reactions like PCR or 3SR, the detection threshold of infectious RNA in human sera could be dropped to concentrations of 10^{-18} M (Walter, Schwille and Eigen, 1996; Oehlenschläger, Schwille and Eigen, 1996). The method is useful in that it allows for simple quantification of initial infectious units in the observed samples. The isothermal Nucleic Acid Sequence-Based Amplification (NASBA) technique enables the detection of HIV-1 RNA in human blood-plasma (Winkler, Bieschke and Schwille, 1997). The threshold of detection is presently down to 100 initial RNA molecules per milliliter, and possibly much fewer in the future, by amplifying a short sequence of the RNA template (Schwille, Oehlenschläger and Walter, 1997). The NASBA method was combined with FCS, thus allowing the online detection of the HIV-1 RNA molecules amplified by NASBA (Oehlenschläger, Schwille and Eigen, 1996). The combination of FCS with the NASBA reaction was performed by introducing a fluorescently labeled DNA probe into the NASBA reaction mixture *at nanomolar concentrations*, hybridizing to a distinct sequence of the amplified RNA molecule. The specific hybridization and extension of this probe during the amplification reaction resulted in an increase of its diffusion time and was monitored online by FCS. Consequently, after having reached a critical concentration on the order of 0.1 to 1.0 nM (the threshold for single-photon excitation / FCS detection is ~0.1 nm), the number of amplified RNA molecules could be determined as the reaction continued its course. Evaluation of the hybridization/extension kinetics allowed an estimation of the initial HIV-1 RNA concentration, which was present at the beginning of amplification. The value of the initial HIV-1 RNA number enables discrimination between positive and false-positive samples (caused, for instance, by carryover contamination). Plotted in a reciprocal manner, the slopes of the correlation curves in the HIV-positive samples drop because of the slowing down of diffusion after binding to the amplified target. This possibility of sharp discrimination is essential for all diagnostic methods using amplification systems (PCR as well as NASBA).

The quantification of HIV-1 RNA in plasma by combining NASBA with FCS may be useful in assessing the efficacy of anti-HIV agents, especially in the early infection stage when standard ELISA antibody tests often display negative results. Furthermore, the combination of NASBA with FCS is not restricted only to the detection of HIV-1 RNA in plasma. Though HIV is presently a particularly common example of a viral infection, the diagnosis of Hepatitis (both B and C) remains much more challenging. On the other hand, the number of HIV, or HBV, infected subjects worldwide is increasing at an alarming rate, with up to 20% of the population in parts of

Africa and Asia being infected with HBV. In contrast to HIV, HBV infection is not particularly restricted to the high-risk groups.

Multi-photon (MPE) NIR excitation of fluorophores--attached as labels to biopolymers like proteins and nucleic acids, or bound at specific biomembrane sites-- is one of the most attractive options in biological applications of FCS. Many of the serious problems encountered in spectroscopic measurements of living tissue, such as photodamage, light scattering and auto-fluorescence, can be reduced or even eliminated. FCS can therefore provide accurate *in vivo* and *in vitro* measurements of diffusion rates, "mobility" parameters, molecular concentrations, chemical kinetics, aggregation processes, labeled nucleic acid hybridization kinetics and fluorescence photophysics/ photochemistry. Several photophysical properties of fluorophores that are required for quantitative analysis of FCS in tissues have already been widely reported. Molecular "mobilities" can be measured by FCS over a wide range of characteristic time constants from $\sim 10^{-3}$ to 10^3 ms.

Novel, two-photon NIR excitation fluorescence correlation spectroscopy tests and preliminary results were obtained for concentrated suspensions of live cells and membranes. Especially promising are further developments employing multi-photon NIR excitation that could lead, for example, to the reliable detection of cancers using NIR-excited fluorescence. Other related developments are the applications of Fluorescence Cross-Correlation Spectroscopy detection to monitoring *DNA- telomerase interactions*, DNA hybridization kinetics, ligand-receptor interactions and HIV-HBV testing. Very detailed, automated chemical analyses of biomolecules in cell cultures are now also becoming possible by FT-NIR spectroscopy of single cells, both *in vitro* and *in vivo*. Such rapid analyses have potentially important applications in cancer research, pharmacology and clinical diagnosis.

2.7. Near Infrared Microspectroscopy, Fluorescence Microspectroscopy and Infrared Chemical Imaging of Single Cells

Novel methodologies are currently being evaluated for the chemical analysis of embryos and single cells by Fourier Transform Infrared (FT-IR), Fourier Transform Near Infrared (FT-NIR) Microspectroscopy, Fluorescence Microspectroscopy. The first FT-NIR chemical images of biological systems approaching 1micron ($1\mu m$) resolution were recently reported (Baianu, 2004; Baianu et al 2004). FT-NIR spectra of oil and proteins were obtained under physiological conditions for volumes as small as $2\mu m^3$. Related, HR-NMR analyses of oil contents in somatic embryos are also presented here with nanoliter precision. Therefore, developmental changes may be monitored by FT-NIR with a precision approaching the *picogram* level if adequately calibrated by a suitable primary analytical method. Indeed, detailed chemical analyses are now becoming possible by FT-NIR Chemical Imaging/ Microspectroscopy of single cells. The cost, speed and analytical requirements are fully satisfied by FT-NIR spectroscopy and Microspectroscopy for a wide range of biological specimens. FT-NIR \Microspectroscopy and Chemical Imaging were also suggested to be potentially important in functional genomics and proteomics research (Baianu et al 2004) through the rapid and accurate detection of high-content microarrays (HCMA). Multi-photon (MP), pulsed femtosecond laser NIR Fluorescence Excitation techniques were shown to be capable of *single molecule detection* (SMD). These powerful microspectroscopic techniques allow for most sensitive and reliable quantitative analyses to be carried out both *in vitro* and *in vivo*. In particular, MP NIR excitation for Fluorescence Correlation Spectroscopy (FCS) allows not only *single molecule detection*, but also non-invasive monitoring of molecular dynamics and the acquisition of high-resolution,

submicron imaging of *femtoliter* volumes inside living cells and tissues. Such novel, ultra-sensitive and rapid NIR/FCS analyses have therefore numerous potential applications in biomedical research areas, clinical diagnosis of viral diseases, cancers and also in cancer therapy.

3. Mapping the Interactome Networks

Mapping protein-protein interaction networks, or charting the global interaction maps, that correspond through translation to entire genomes is undoubtedly useful for understanding cellular functions, especially when such databases can be integrated into a wide collection of biologically relevant data. A prerequisite for any '*ab initio'* determination of a selected protein interactome network is to clone the open reading frames (ORFs) that encode each protein present in the selected network. Note, however, that all current analyses involve the assumption of a *model* together with some 'hidden', or implicit, *assumptions* about sampling, 'noise' levels, or uniformity/ accuracy in the database, and therefore, the '*ab initio'* claim is subject to the restrictions imposed by such additional assumptions. More than 20,000 of publicly accessible, full ORF clones have been already collected for human and mouse protein-coding genes in the Mammalian Genome Collection (MGC; http://mgc.nci.nih.gov). This community resource enables the next stages of human interactome analysis that will be directed at obtaining a reliable map of the entire human protein interactome. An additional, 12,500 ORFs are now available from the Dana Farber Cancer Institute in Boston (USA) from high-throughput, yeast two-hybrid (Y2H) analyses. A disconcerting aspect of the latest human (partial) interactome studies by different methods is the little apparent overlap of the new human interaction datasets with each other and/or with previously reported data. This aspect will be further addressed later in this section; the principal cause for the lack of overlap is likely to be caused by the low (<20%) overall coverage of the protein-protein interactions selected in such studies. A possible solution to this problem has been suggested (Warner et al 2006): several groups cooperating to produce 'networks of networks', constructed from separate—but coordinated—interaction mapping projects, 'each of which would target a specific functionality related subset of proteins and interactions'. A more effective solution would be, however, to increase the throughput, accuracy and reliability of PPI data through improved technologies (such as FCCS, or other techniques already proposed in Section 2.6, for example) , reduce significantly the cost of such analyses, as well as improve the models employed for data analysis. Examples of improved modeling tools for this purpose, such as logical, ontological genetics and categorical ones, that are also appropriate for assembling the '*networks of networks…*' as in the previous approach suggested by Warner et al. (2006), were presented above in Section 1, and are described in further detail in a recent report (Baianu et al 2006) and also in two forthcoming publications (Baianu and Poli, 2007; Baianu et al 2007).

Interactome network studies are currently undertaken by a number of international research teams in the US, Europe and Japan (CSH/WT, 2006; Warner et al 2006). These studies are currently undertaken only for Interactome subnetworks because of both technique and funding limitations. The organisms studied are: yeast (*Saccharomyces cerevisiae*), worm (*Caenorhabditis elegans*), fruitfly (*Drosophila melanogaster*) and humans. Proteome networks were investigated for several, specific, biological processes such as: DNA degradation, ubiquitin conjugation, multivesicular formation, intracellular membrane traffick, signal transduction/ TNFα tumor necrosis and NFκB mediated pathways, and early stages of T-cell signaling (for a brief summary note the recent review by Warner *et al* 2006, and references cited therein). Such challenging studies face both methodological problems such as limited sampling (Han *et al* 2006) and consideration of only pairwise ('binary') protein-protein interactions, and also the more

serious technical problem of false-positive interactions in the presence of a significant 'noise' levels associated with the experimental technologies and design currently employed in such studies. Such limitations should be borne in mind (Han et al 2006) when global topology predictions are made for the whole interactome based on partial, incomplete data obtained for subnetworks that may contain less than 20% of the entire interactome network. On a more optimistic note are the recent attempts at comparing the cancer protein, human interactome (sub) networks with normal human interactome networks that involve multiple protein-protein interactions (Jonsson and Bates, 2006). The latter studies reduced the 'noise' level in the human protein interaction data by employing an orthology-based method described previously by Jonsson et al. (2006). This method claims to reduce the 'noise' level in protein-interaction (PPI) data by identifying putative interactions based on homology to experimentally determined interactions in a range of different species; both the DIP (Salwinsky et al 2004) and the MIPS, Mammalian Protein—Protein Interaction (Pagel et al 2005) databases were utilized. Furthermore, the complete interactome data set that was employed is available as Supplementary Material from *loc. cit.* The conclusions was drawn that cancer proteins have an increased frequency of protein-protein interactions in comparison with the proteins that were studied in normal cells, and this was interpreted as evidence "*indicating an underlying evolutionary pressure to which cancer genes, as genes of central importance are subjected.*" It remains to be seen, however, if human interactome studies-- which occur with increasing frequency-- have indeed overcome the sampling objections raised by Han et al. (2006). The more extensive interactome data and analysis—though still quite limited- that has been reported to date is readily available and includes the following: Y2H (partial data-based) interactome maps for *C. elegans* (Li et al 2004) and *Drosophila melanogaster* (Giot et al 2003; Formstecher et al 2005), and also proteome maps obtained by co-affinity purification followed by mass spectrometry analysis in yeast- *Saccharomyces cerevisiae* (co-AP/MS: Gavin et al 2002; Ho et al 2002; Han et al 2004). The reports on the microbial transcriptional regulation network of *Escherichia coli* (Shen-Orr et al 2002) and on *Helicobacter pylori* protein complexes in the proteome map (Terradot et al 2004) are also worthile mentioning in this context. A first-draft of the human interactome has also been reported (Lehner and Fraser, 2004); although this human interactome map does not seem to have been included in the computational investigations of Han et al. (2006), it remains to be verified, or validated, by further extensive studies with improved technology and adequate models for a more comprehensive data analysis. The comprehensive two-hybrid analysis for exploring the protein interactome network was previously reported by Ito et al. (2001). Alternative interaction mapping strategies have also been developed over the last five years. An example is the tandem affinity purification (TAP) in conjunction with liquid chromatography tandem mass spectrometry (LC-MS/MS; see, for example, Gavin et al 2006). Such methods have, however, both advantages and limitations. An interesting, new approach to the determination of protein complexes has been developed that involves a combination of fluorescence spectroscopy with peptide microarrays (Stoevesandt, *cited in* Warner 2006); this methodology was then applied to investigate T-cell signaling.

4. Cell Cyclins Expression and Modular Cancer Interactome Networks

Carcinogenesis is a complex process that involves dynamically inter-connected biomolecules in the intercellular, membrane, cytosolic, nuclear and nucleolar compartments that form numerous inter-related pathways referred to as networks. One such family of pathways contains the cell cyclins. Cyclins are often overexpressed in cancerous cells (Dobashi et al 2004).

Our novel theoretical analysis based on recently published studies of cyclin signaling, with special emphasis placed on the roles of cyclins D1 and E, suggests novel clinical trials and rational therapies of cancer through re-establishment of cell cycling inhibition in metastatic cancer cells.

4.1. Cyclins

Cyclins are proteins that link several critical pro-apoptotic and other cell cycling/division components, including the tumor suppressor gene TP53 and its product, the Thomsen-Friedenreich antigen (T antigen), Rb, mdm2, c-Myc, p21, p27, Bax, Bad and Bcl-2, which all play major roles in carcinogenesis of many cancers. Cyclin-dependent kinases (CDK), their respective cyclins, and inhibitors of CDKs (CKIs) were identified as instrumental components of the cell cycle-regulating machinery. CDKs are enzymes that phosphorylate several cellular proteins thus 'fueling' the sequential transitions through the cell division cycle. In mammalian cells the complexes of cyclins D1, D2, D3, A and E with CDKs are considered motors that drive cells to enter and pass through the "S" phase. Cell cycle regulation is a critical mechanism governing cell division and proliferation, and is finely regulated by the interaction of cyclins with CDKs and CKIs, among other molecules (Morgan et al 1995).
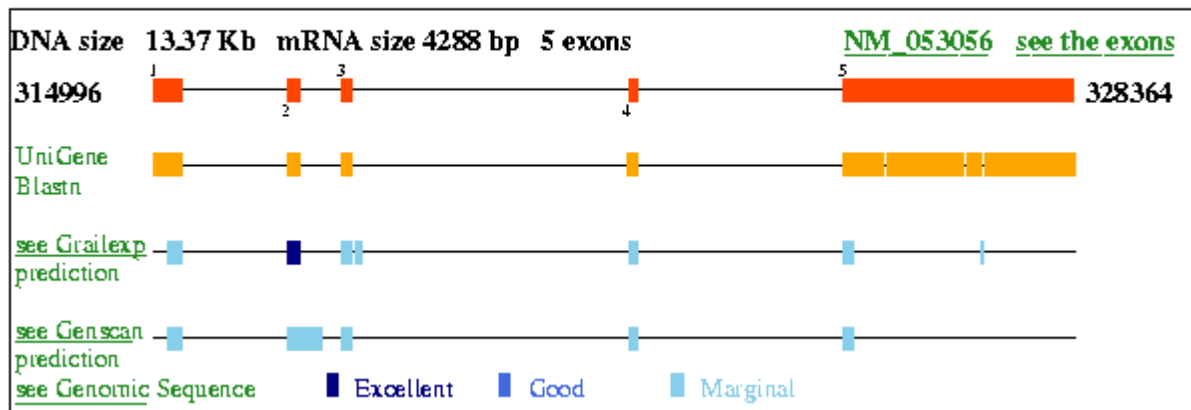
Fig. 1: Gene database of Cyclin-D1, from the PBD website: http://www.dsi.univ-paris5.fr/genatlas/fiche.php?symbol=CCND1

It was also reported that CDKs have another key role –the coordination of cell cycle progression with responses to possible DNA-damage that could, if unchecked or unfixed, lead to a lack of genomic integrity marking the onset of cell disease including cancers (Huang et al 2006 in *Science*). The S-phase is thought to be the most vulnerable interval of the cell cycle because during this interval all of 3 billion DNA bases of the human genome must be replicated precisely in the sense of 'carbon copies' being made of the existing DNA strands, without any breaks in the sequence or base substitutions of the copied/replicated strands. Therefore, this correct replication process controls the cell's survival, especially under genotoxic conditions such as those caused for example by mutagens or X-ray/ γ–radiation. Furthermore, Huang et al. (2006) reported that CDK mediated the phosphorylation of the FOXO1 transcriptional activator of the proapoptotic genes during the S-phase; when DNA damage occurs either before or during the S-phase, a complex network is activated in the cell which 'silences' CDK thereby either delaying or

stopping/arresting the cell cycle progression. This may allow the cell to repair the DNA damage by recombination involving BRCA2 and survive. However, if this is not possible because the DNA damage was too great/irreparable, then FOXO1 would trigger apoptosis (cell death). It was proposed that during the unperturbed (normal) S-phase CDK2 phosphorylates FOXO1 at the Serine[249] residue in the cell nucleus, which then results in the transfer and sequestering of the FOXO1 in the cytoplasm, where it is well--separated from the proapoptotic genes, the 'target' of FOXO1 action. Moreover, the CDK-mediated phosphorylation of BRCA2 during the unperturbed S-phase renders inactive the DNA recombination. On the other hand, when DNA becomes damaged, CDK2 is inhibited through the Cdc25A pathway, with the consequence of a dephosphorylated FOXO1 which then remains in the cell nucleus and is able to activate the proapoptotic genes, unless BRCA2 is able to induce DNA recombination and repair in time to prevent apoptosis. The steps that follow are then as explained above: either DNA repair and continued cell cycling, or apoptosis induced by FOXO1. There are still several important questions regarding the entire process that need to be answered before the FOXO1 and CDK2 mechanisms of action can be translated into successful clinical trials based on such knowledge.

A positive correlation has been noticed between overexpression of several cell--cycle proteins and unfavorable prognoses and outcomes in several different cancer types (van Diest et al 1995; Handa et al 1999; Fukuse et al 2000). In human lung tumors and soft tissue sarcomas, it has recently been discovered that cyclin A/cdk2 complex expression and kinase activity were reliable predictors of proliferation and unfavorable prognosis, thereby further substantiating the epidemiological factors of cyclin signaling (Dobashi et al 2003; Noguchi et al 2000).

4.2. The p27 and p21 Proteins

The proteins p27 and p21 are implicated in cyclin regulation and cancer development (Fig. 3). Mouse embryonic fibroblasts that were deficient for p27 and p21 were found to contain less cyclin D1 and D2 (Cheng et al 1999) as well as cyclin D3 (Bagui et al 2000) than controls. Similarly, mammary glands of p27-deficient mice were shown to possess decreased cyclin D1 levels (Muraoka et al 2001). It has been demonstrated *in vivo* that p27 is necessary for maintaining proper levels of cyclins D2 and D3, and this dependency on p27 is common to a wide variety of cells/tissues *in vivo*. Regarding the molecular interaction between p27 and D-cyclin, CDK4 is a clear candidate as a mediating molecule (Bryja et al 2004). Cells employ CDK4/6– cyclin D complexes to flexibly titrate p27 from the complexes containing CDK2, and thereby they control their proliferation. However, mutual dependency between cyclin D and p27 serves also some yet unidentified function in differentiation-related processes. Thus, loss of p27 not only causes unrestricted growth due to inefficient inhibition of CDK2–cyclin E/A, but may also elicit a decrease in levels of D-type cyclins, resulting in differentiation defects. Upon ablation of cyclin D, cells lose their ability to titrate p27 from CDK2–cyclin A/E complexes and proliferation is suppressed. However, defects in differentiation caused by the absence of D-cyclin are reminiscent to defects produced by the absence of p27 (Bryja et al 2004). When the changes in levels of p27 and/or D-type cyclins occur, an equilibrium alteration could result between proliferation/differentiation processes that may in the end result in tumorigenesis (Bryja et al 2004).

4.3. D1 vs. E- Cyclins

The D-type and E-type cyclins control the G1 → S phase transition during normal cell cycling and are important components of steroid- and growth factor-induced mitogenesis in

breast epithelial cells (Sutherland and Musgrove, 2004). Cyclin D1 null mice are resistant to breast cancer that is induced by the *neu* and *ras* oncogenes, which suggests a pivotal role for cyclin D1 in the development of some mammary carcinomas (Sutherland and Musgrove, 2004). Cyclin D1 and E1 are usually overexpressed in breast cancer, with some association with adverse outcomes, which is likely due in part to their ability to confer resistance to endocrine therapies. The consequences of cyclin E overexpression in breast cancer are related to cyclin E's role in cell cycle progression, and that of cyclin D1 may also be a consequence of a role in transcriptional regulation (Sutherland and Musgrove, 2004). One critical pathway determining cell cycle transition rates of G1 → S phase is the cyclin/cyclin-dependent kinase (Cdk)/ p16Ink4A/ retinoblastoma protein (pRb) pathway (Sutherland and Musgrove, 2004). Alterations of different components of this particular pathway are very ubiquitous in human cancer (Malumbres and Barbacid, 2001). There appears to be a certain degree of tissue specificity in the genetic abnormalities within the Rb pathway. A model relating Rb to cyclin control in the overall scheme of pro-apoptotic behavior is shown below (Fig. 4). In breast cancer these abnormalities include the over-expression of cyclins D1, D3 and E1, the decreased expression of the p27Kip1 CKI and p16Ink4A gene silencing through promoter methylation. These aberrations occur with high frequency in breast cancer, as each abnormality occurs in ~40% of primary tumors. This fact implicates a major role for the loss of function of the Rb pathway in breast cancer. Cyclin D1 is the product of the *CCND1* gene and was first connected to breast cancer after localization of the gene to chromosome 11q13, a region commonly amplified in several human carcinomas, including ~15% of breast cancers (Ormandy et al 2003). The fact that cyclin D1 was overexpressed at the mRNA and protein levels in 50% of primary breast cancers have caused cyclin D1 to be considered one of the most commonly over-expressed breast cancer oncogenes (Gillett et al 1994; Alle et al 1998).

Fig. 3. Pro-Apoptotic Cancer Cycling Model (modified from Aguda et al 2003).

Mvc

E2F

Rb

p53

MDM2

ARF

Bax

Bcl-2

p21

CycE/cdk4

DISC

Apoptosome

Exec. Caspases

Cdc25A

CycD/cdk2

p27
unP

T antigen

PTEN
(inactivated)

Cancer

GTP

GEFs

GDP

PI-3
kinase/Akt

PIP$_2$→PIP$_3$

H-Ras-GDP

H-Ras-GTP
(active)

Raf

MAP kinases

GDIs

RalGDS

RalA/B
activation

P$_i$
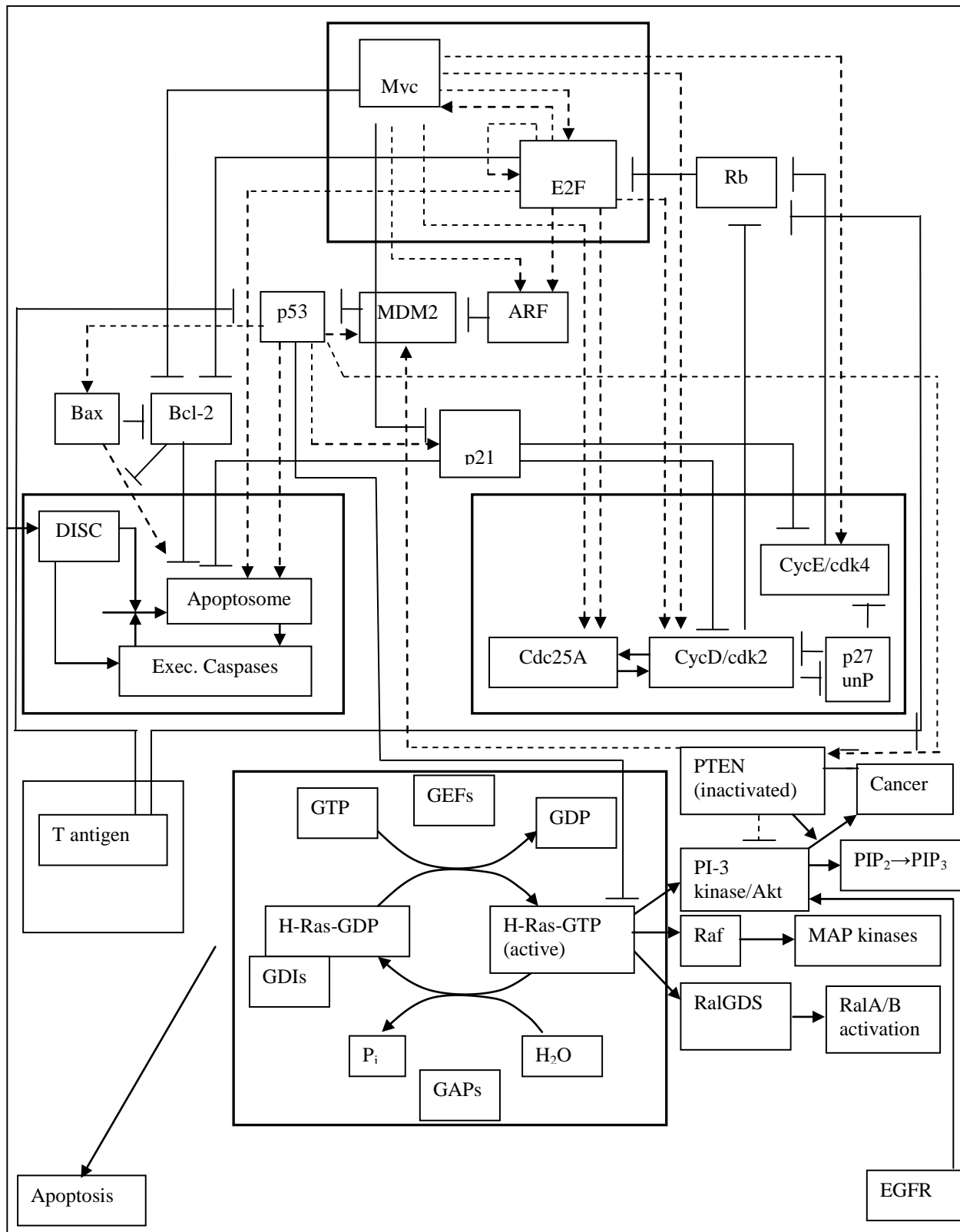
H$_2$O

GAPs

Apoptosis

EGFR

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Fig. 3. Pro-Apoptotic Cancer Cycling Model (modified from Aguda et al 2003).

Although cyclin E1 locus amplification is rare in breast cancer, the protein product is overexpressed in over 40% of breast carcinomas (Loden et al 2002). Cyclin D1 is predominantly overexpressed in ERC tumors, and cyclin E overexpression is confined to ER¡ tumors (Gillett et al 1994; Alle et al 1998; Loden et al 2002). The overexpression of several cell cycle regulators has been strongly associated with apoptotic-like behavior, as well as frank apoptosis, in cancer cells, which include c-Myc, E2F-1 and HPV. Apoptosis and its connection to cell cycle-related proteins is of interest therapeutically, as these types therapies could ultimately lead to the cancer cell annihilation via apoptosis. Recently, a shift has occurred, changing the focus of chemotherapy from exploration of agents that cause cell growth arrest to those that favor apoptosis.

FGFR Tyrosine Kinases

Fibroblast growth factor receptor (FGFR) tyrosine kinases have recently been studied as they relate to intracellular signaling and their effects on pRb, and are of interest to the field of cancer biology. Overexpression of FGFR tyrosine kinases has been found in many human breast carcinomas and has been associated with poor clinical prognosis (Koziczak et al 2004). Fibroblast growth factor receptors (FGFRs) are glycoproteins composed of extracellular immunoglobulin (Ig)- like domains, a hydrophobic transmembrane region and a cytoplasmic moiety that contains a tyrosine kinase domain (Koziczak et al 2004). When active, FGFRs stimulate tyrosine phosphorylation, as well as activation of several signaling molecules: Shc, PI3K, Src, PLCg, Crk, SH2 domain containing phosphatase-2 (SHP-2), p38, STAT1/3 and FGFR substrate 2 (FRS2) (Klint and Claesson-Welsh, 1999). Treatment of tumor cells with the FGFR tyrosine kinase inhibitor leads to a reduction in pRb phosphorylation on serine 795, a site known to be phosphorylated by the cyclin D/cdk4 complex (Koziczak et al 2004). FGFR signaling may in fact promote cell proliferation by upregulating cyclin D levels. This idea is supported by the fact that ectopic cyclin D1 expression is able to rescue the FGFR inhibitor-mediated antiproliferative effect (Koziczak et al 2004). Using a cyclin D1 reporter gene, Koziczak et al. (2002) found that FGFR inhibitor caused a significant reduction in promoter activity, and was reflected in an overall decrease in cyclin D1 mRNA levels.

A recent study employed p27-deficient mice to investigate the significance of p27 for the metabolism of D-type cyclins in differentiated cells (Bryja et al 2004). The absence of p27 resulted in decreased cyclins D2 and/or D3 levels in several organs. The drop in cyclin D levels that was due to the absence of p27 equaled the amount of cyclin D physically associated with p27 animal controls. This indicates the possibility that it is the fraction of p27-associated cyclin D that determines the response to p27 deficiency. Cells in which the D-type cyclin level is dependent on p27 do not up-regulate their CDK2 and CDK4 activities upon deactivation of p27 (Fig. 5). Moreover, these cells have a negligible amount of p27 bound to CDK2 and/or cyclin A/E under non-cancerous conditions (Bryja et al 2004). These findings point to the existence of two roles for p27: regulation of the cell cycle through inhibition of CDKs, and participation in the establishment or maintenance of the differentiated status that is achieved in conjunction with D-cyclins (Bryja et al 2004).
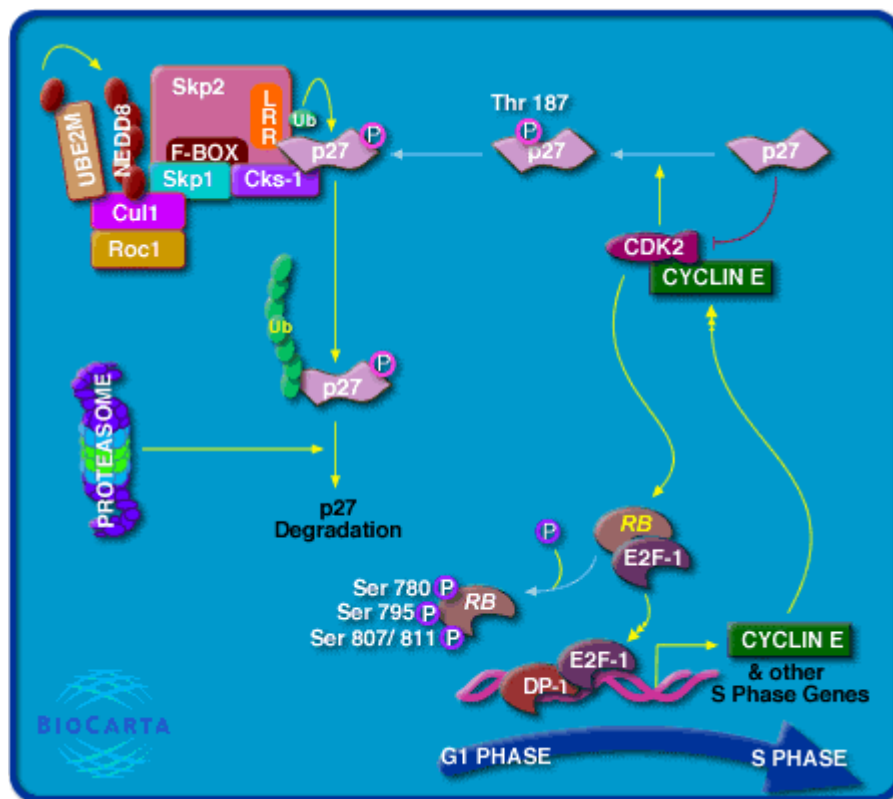
Fig. 4. Regulation of p27 Phosphorylation during Cell Cycling
(http://cgap.nci.nih.gov/Pathways/BioCarta/h_p27Pathway)

Ubiquitin

The regulation of protein stability via the ubiquitin–proteasome pathway is critical to the comprehension of the biomolecular basis of cancer development. However, ubiquitin modification of substrates signals many cellular processes (besides proteolysis) that are also important for cancer development. Interestingly, many breast cancer proteins studied by clinical researchers are involved in these specific ubiquitin pathways. These proteins include cyclins, CDK inhibitors and the SCF in cell cycle control, the breast and ovarian cancer suppressor BRCA1-BARD1, ErbB2/HER2/Neu and its ubiquitin ligase c-Cbl , as well as and the estrogen receptor and its target, Efp.

One function of the ubiquitin–proteasome proteolysis pathway is to label proteins for rapid degradation. It consists of four enzymes: a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), a ubiquitin ligase (E3) and the 26S proteasome (Hershko and Ciechanover, 1998). E1 binds to and activates ubiquitin in an ATP-dependent manner through a thiolester bond and then transfers ubiquitin to an E2 enzyme. E2 then transfers ubiquitin to a lysine residue in the substrate via a terminal isopeptide bond through E3. E3 is a scaffold protein that bridges in the substrate and the ubiquitin-bound E2. The resultant covalent bonds of the ubiquitin ligations form polyubiquitinated conjugates that are quickly found and digested by the 26S proteasome. Understanding these pathways may provide many critical clues toward the

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development of novel diagnostic tools and treatments for cancer patients (Ohta and Fukuda, 2004).

In the past decade researchers have identified important functional roles for the D- and E-type cyclins in the evolution of human breast cancers. These genes are among the most commonly overexpressed genes in breast cancer, being overexpressed in the early phases of disease and having proven oncogenic effects on mammary epithelial cells both *in vitro* as well as *in vivo*. Their established role in CDK activation and Rb pathway regulation has directed scientific attention toward aberrant cell cycling as the basis of oncogenic potential. More recent data on the role of different G1 cyclins in the areas of differentiation, chromosome stability and transcriptional regulation indicate that their role in breast cancer is much more complex than initially predicted.   Further investigations may yield a more complete understanding of the role of these cyclins regarding the biomolecular basis and pathophysiology of breast cancer, with significant potential benefits clinically, through the identification of novel markers of prognosis and therapeutic responsiveness and potential new targets for innovative clinical intervention.

5. Biomedical Applications of Microarrays in Clinical Trials

5.1. Microarray Applications to Gene Expression: Identifying Signaling Pathways

Changes in homeostasis can be followed through various experimental strategies that monitor gene expression profiling, for example, by employing high-throughput microarray technology. This section discusses briefly the successful use of microarray technology in RNA expression studies aimed at identifying signaling pathways that are regulated by key genes implicated in carcinogenesis/ tumorigenesis. A primary objective of tumor-profiling experiments is to identify transcriptional changes that may be the cause of the transition from the normal to the tumor phenotype. Such changes may, however, occur also as a consequence of various neoplastic transformation(s). More importantly, this approach may allow the identification of molecular fingerprints that can be utilized for the classification of different tumor types, and are therefore valuable diagnostic molecular tools in cancer patients. For example, Alizadeh et al. (2000) have successfully used such an approach to identify molecularly distinct subclasses of diffuse large B-cell lymphoma that could not be distinguished by conventional diagnostic tools. In another study, a molecular fingerprint comprising approximately 50 genes has been isolated from a total of over 6,000, and this fingerprint can reliably differentiate between acute myeloid leukemia and acute lymphoblastic leukemia Golub et al (1999). The approach requires, however, multiple independent experiments with several large groups of samples in order to enable one to reliably and reproducibly separate the biologically relevant changes from false ones that may occur as a result of the genetic heterogeneity between individual samples from the same tumor, for example. The two examples quoted above were able to reproducibly identify tumor type-specific molecular determinants through multiple experiments with various tissue samples.

5.1.1. Identification of Specific Transcriptional Targets in Cancer

A different experimental approach to the one presented above is, however, needed for identifying specific targets such as defined genes that are implicated in cancer progression; this involves monitoring changes in transcriptional profile that occur as a result of modulation of the expression level of the defined gene, or genes, selected for such studies. The altered expression profile can be viewed as a 'blueprint' by which the defined gene controls its cellular function.

The transcriptional profiles are thus employed to define *downstream signaling pathways* that have been previously validated through other techniques such as differential display Tanaka et al (2000) and serial analysis of gene expression Yu et al. (1999). This approach combined with microarray technology allows the simultaneous identification of all potential targets. Its only drawback is the reliance upon the prior knowledge of the selected genome for such investigations. The caveat is, however, that the investigator who employs this approach needs also to devise additional experiments in order to confirm that genes identified with the microarray are indeed *physiologically relevant* targets.

5.1.2. Identification of Downstream Transcriptional Targets of the BRCA1 Tumor-Suppressor Gene

The breast and ovarian cancer susceptibility gene BRCA1 is probably the most studied gene in the breast cancer field because of its clinical significance and multiple functions. BRCA1 was shown to be mutated in the germline of women with a genetic predisposition to either breast or ovarian cancer Mikki et al (1994). Most mutations identified reported have resulted in the premature truncation of the BRCA1 protein. BRCA1 is known to encode a 1863 amino acid phosphoprotein that is predominantly localized to the nucleus, presumably with a unique function. Protein sequence analysis identified a C-terminal BRCT motif, which was then postulated to play a role in cell cycle checkpoint control in response to DNA damage Koonin EV, Altschul and Bork (1996). Consistent with this postulated role, BRCA1 becomes hyperphosphorylated in response to various agents that damage DNA such as γ/X--ray-irradiation, an effect that was reported to be partially mediated by chk2 kinases (Lee et al. 2000). Furthermore, BRCA1 has been shown to be implicated in at least three functional pathways:

A. Mediating the cellular response to DNA damage,
B. Acting as a cell cycle checkpoint protein, and
C. Functioning in the regulation of transcription.

However, the physiological significance of such BRCA1 actions as well as their relationships with the function of BRCA1 as a tumor-suppressor gene still remain to be defined. Further details are presented next.

The BRCA1-BARD1 Ubiquitin Ligase

As already stated above, the BRCA1 gene encodes a 1863-amino-acid protein (Miki et al 1994) that consists of a RING-finger domain in its terminal N-region, a region that includes a nuclear localization signal and a domain that binds to many cellular proteins, and tandem BRCT domains in its C-terminal region. BRCA1 is associated with a diverse range of biological processes, such as DNA repair, cell cycle control, transcriptional regulation, apoptosis and centrosome duplication. Thus, a specific role has already been postulated for BRCA1 in transcriptional regulation. The C-terminal domain of BRCA1 was reported to contain a potent transactivation domain when this was fused to a heterologous DNA binding motif (Monteiro, August and Hanafusa, 1996). The oligonucleotide array-based expression profiling described above in Section 2.2 was employed by Haber (2000) in collaboration with Affymetrix Co. to identify the downstream transcriptional targets of the BRCA1 tumor-suppressor gene in order to define its function (Harkin et al 1999).

The only known biochemical function of BRCA1 is its E3 ubiquitin ligase activity. The N-terminal RING finger domain of BRCA1 interacts with another conformationally similar RING finger protein, BARD1 (Wu et al 1996; Brzovic et al 2001), that also contains an N-terminal RING domain and C-terminal BRCT domains (Wu et al 1996). BRCA1 attains high ubiquitin ligase activity when bound to BARD1 as a heterodimer (Hashizume et al 2001). Importantly missense mutations in the RING-finger domain of BRCA1 found in familial breast cancer all eradicate the ubiquitin ligase activity of BRCA1-BARD1 (Hashizume et al 2001; Ruffner et al 2001; Brzovic et al 2003). This fact suggests a *strong link between BRCA1 ligase activity and its function as a tumor suppressor*. The analysis of ubiquitin ligase activity of RING-domain mutations is important not only for the investigation of the biological function of BRCA1, but also to be able to predict a specific patient's propensity for cancer, which may influence the determination of the need for prophylactic surgery.

Besides enhancing BRCA1's ubiquitin ligase activity, BARD1 is also critical for BRCA1 stability *in vivo* (Hashizume et al 2001; Joukov et al 2001; Xia et al 2003). Loss of BARD1 leads to a phenotype similar to that of the loss of BRCA1, that is, early embryonic lethality/ chromosomal instability (McCarthy et al 2003). Moreover, germline mutations of BARD1 are found in breast and ovarian cancer patients (Thai et al 1998). Although ubiquitin ligase activity may be significant for the role of the BRCA1 gene as a tumor suppressor, the way the activity contributes to BRCA1's biological function remains unknown. Two issues exist that are critical to the elucidation of the role of the BRCA1-BARD1 ubiquitin ligase: the type of polyubiquitin chain built by BRCA1-BARD1 (and its consequences), and the specific identity of its substrates.

The following reported observations provide only indirect, additional clues to the tumor-suppressor gene function of BRCA1. Germline mutations of BRCA1 were reported for half of breast-ovarian cancer pedigrees and for approximately 10% of women with early onset of breast cancer, uncorrelated with their family history (Fitzgerald et al 1996). It was also shown in other studies that somatic inactivation of BRCA1 is rare in sporadic breast cancers (Futreal P, Liu Q and Shattuck-Eidens 1994), and mutations were reported for approximately 10% of sporadic ovarian cancers, therefore suggesting potentially distinct genetic mechanisms for sporadic, breast and ovarian cancers (Berchuk et al 1998). The reduced BRCA1 protein expression reported for the majority of sporadic breast cancers indicates that *epigenetic mechanisms* (see also Section 6) may also play a significant role in regulating the BRCA1 expression (Wilson et al 1999). Furthermore, a defect was reported in the transcription-coupled repair of oxidative-induced DNA damage in mouse embryo fibroblasts with attenuated BRCA1 function (Gowen et al 1998); this observation would suggest that BRCA1 plays a more general role in mediating the cellular response to DNA damage. Thus, BRCA1 has also been reported to be involved in cell cycle checkpoint control, by becoming hyperphosphorylated during late G_1 and S cell phases, and then changing to transiently dephosphorylated early after the M phase (Ruffner and Verma, 1997). Moreover, the BRCA1 overexpression has been reported to induce a G_1/S arrest in human colon cancer cells (Somasundaram et al, 1997). By comparison with the cancer regulation model in Figure 3, it seems very significant for oncogenesis that BRCA1 is *physically associated* with the transcriptional regulators *p*53 (Ouichi et al 1998), CtIP (Yu et al 1998), c-Myc (Wang et al 1998), as well as the histone deacetylases HDAC1 and HDAC2 (Yarden and Brody 1999). The physical association of BRCA1 with c-Myc acquires special significance as c-Myc seems to be involved in controlling telomerase activity, whereas p53 is involved in DNA-repair, cell-cycling and apoptosis. Therefore, in the simplified model presented in Figure 3, one should add the BRCA1 links to both p53 and c-Myc in order to facilitate an understanding of the BRCA1 possible roles in oncogenesis.

5.1.3. Selecting Gene Expression Systems

There are several related problems in studying gene function by expression profiling. For example, it has been often reported to be difficult to generate cell lines that overexpress genes such as BRCA1, or p53, because their forced overexpression can lead either to growth suppression or apoptosis (as shown for example in Figure 3, and at the end of the previous section). However, in the case of BRCA1, it was reported that the *tet-off* inducible expression system (Gossen and Bujard 1992) can be utilized to generate cell lines with highly regulated inducible expression of BRCA1 (Harkin et al, 1999). This inducible expression system introduces into the cells a chimeric transactivator; the latter consists in the *tet* repressor fused to the VP16 transactivation domain. This chimeric transactivator is inactive in the presence of tetracycline, whereas in the absence of tetracycline it can bind to promoters that contain the *tet* operator sequence; the latter sequence is then utilized to drive the expression of BRCA1. This expression system has a major advantage in that it allows the change in just one parameter involved in the induction of BRCA1. The BRCA1 induction in one population is the only difference between the genetic backgrounds of the two populations that are being compared by oligonucleotides arrays. A number of BRCA1 transcriptional targets can thus be identified with Affymetrix oligonucleotides arrays, and among these, the stress and DNA damage-inducible gene *GADD45* was the gene that exhibited the greatest degree of differential signal intensity (Harkin et al, 1999). The specific target genes thus identified were also verified by Northern blot or quantitative reverse transcriptase-PCR analysis in order to confirm induction in response to the stimulus, that is, the induction of BRCA1 (Harkin et al, 1999). Total RNA was extracted from cells in which the exogenous BRCA1 was either switched off (+ *tet*) or switched on (– *tet).* Fluorescent images were generated using the Affymetrix human cancer G110 array containing approximately 1,700 genes that were previously reported to be implicated in cancer*;* such fluorescent images were then scanned and analyzed. Two lanes were present in such images that corresponded to individual arrays that were hybridized with biotinylated cRNA probes generated from cells in which exogenous BRCA1 was either induced (+ *tet*) or repressed (- *tet*). Each gene on the array was represented by 16 probe pairs, one being wild-type and one containing a mismatch at the central nucleotide. In such fluorescent images, two genes, GADD45 and ATF3 were identified (and confirmed by Northern blot analysis) as being the *transcriptional targets of the BRCA1 tumor-suppressor gene*. Furthermore, in this BRCA1 study, the induction of GADD45 by BRCA1 was reported to be *correlated with the BRCA1-mediated activation* of the c-jun *N*-terminal kinase/stress-activated protein kinase JNK/SAPK pathway. Significantly, the activation of JNK/SAPK was then shown to be required for the BRCA1-mediated apoptotic cell death in this cell line system. This finding suggests an interesting model for the BRCA1-mediated apoptosis, as presented in some detail in Harkin et al (1999). Most significantly, the experimental approach reported by Harkin et al (1999) was indeed able to defin*e physiologically relevant target genes*.

In another recent report, Yu et al (2001) utilized a modified version of the tet-off inducible expression system to define the downstream transcriptional targets of the *p*53 tumor--suppressor gene (Yu et al 1999). A total of 34 genes were identified that exhibited at least a 10-fold upregulation in response to the inducible expression of *p*53. Somewhat surprisingly, there was a marked heterogeneity of the response when it was evaluated in different cell lines derived from the same tissue of origin. Among the 33 genes studied only nine were found to be induced in a

panel of five unrelated colorectal cell lines, and 17 were induced in a subset; eight were not induced at all in any of the five cell lines examined. This can be interpreted as being due to a high degree of cell type specificity. Furthermore, *p*53 was not absolutely required for induction -- for the majority of the genes identified-- in response to either adriamycin or 5-FU. Therefore, these agents do not seem to act exclusively through *p*53, suggesting that there is inherent redundancy in the majority of signaling pathways. Such inherent redundancy in signaling pathways of cancer, and untransformed, cells might be important in understanding the results of clinical trials in cancer treatment with signal transduction modulators that will be discussed in the next subsection (5.2).

5.2. Clinical Trials with Signal Transduction Inhibitors -- Novel Anticancer Drugs Active in Chemo-resistant Tumors

Recently, there is an increasing number of reports suggesting that human cancers frequently involve pathogenic mechanisms which give rise to numerous alterations in signal transduction pathways. Therefore, novel therapeutic agents that target specific signal transduction molecules or signaling pathways altered in cancer are currently undergoing clinical trials often with remarkable results in cancer treatments of patients in which chemo- and/or radio- therapy resistant tumors have become apparent. For example, several new classes of such anti-cancer drugs are:

- tyrosine/threonine kinase inhibitors, including: STI-571 ('Gleevec', or Imatinib Mesylate), ZD-1839 ('Iressa'), OSI-774, and flavopiridol, which are ATP-site antagonists and have recently completed phase I and phase II trials;

- several other kinase antagonists that are currently undergoing clinical evaluations, including UCN-01 and PD184352;

- other strategies for downmodulating kinase-driven signaling include 17-allyl-amino-17 demethoxygeldanamycin and rapamycin derivatives. Phospholipase-directed signaling may also be modulated by alkylphospholipids.

- Farnesyltransferase inhibitors, originally developed as inhibitors of *ras*-driven signals, may attain activity by affecting other/or additional targets.

- monoclonal antibodies Herceptin and C225.

Signal transduction is an efficient method for fine-tuning the development and modeling of cancer treatments. The following detailed background on clinical trial and signal transduction modulators as novel anticancer drugs summarizes the contents of a recent NCI Report (Sausville, Elsayed, Monga and Kim, 2003).

A. *Tyrosine Kinase Inhibitors:*

1. STI-571, or '**Gleevec', or Imatinib Mesylate**

STI-571 *Main features and action mode*:

- a. Inhibits three kinases: Abl (all forms), PDGFR and c-kit tyrosine kinases;
- b. *Blocks* the Bcr-Abl tyrosine kinase;

c. important in chronic myelogenous leukemia (CML) patients because CML cells have constitutively active Bcr-Abl tyrosine kinase;

d. STI-571 *differentially* inhibited the growth of **p210$^{Bcr-Abl}$** CML and **p185$^{Bcr-Abl}$** CML containing acute lymphoblastic leukemia cells and does **not** affect the normal marrow cells;

e. The effect of STI-571 is exciting because it inhibits c-kit/CD117 positive tumors owing to the paucity of interventions for these chemoresistant tumors. Example: significant response in rapidly progressive gastrointestinal tumors (GIST) and soft-tissue sarcomas that were previously resistant to cytostatic, anticancer drugs when Gleevec is not administered simultaneously with such cytostatics;

f. FDA has approved Gleevec for GIST as well as CML treatments, and is undergoing clinical trials for novel therapeutic strategies of other types of cancer.

2. SU5416

a. This ATP-site antagonist of the vascular endothelial growth factor (VEGF) (Flk1/KDR) receptor was designed following studies of the indolin-2-one pharmacophore and the fibroblast growth factor (FGF) receptor tyrosine kinase domain. A Lineweaver-Burk analysis showed SU5416 to be a competitive inhibitor with ATP for the Flk1/KDR and PDGF receptors (K_i 0.16 µM and 0.32 µM, respectively) (Mohammadi et al 1997; Mendel et al 2000).

b. The first SU5416 clinical trial enrolled 63 patients and administered the drug i.v. biweekly (Rosen et al 1999); at the higher doses, nausea, vomiting, headache and some liver toxicity were noticed;[stable disease of greater than 6 months duration was the only reportable outcome in patients with a variety of advanced diseases (colorectal, lung, renal and Kaposi's sarcoma;

c. Patients with significant progression suffered noticeable increases in vascularity; the occurrence of vascular complications like thrombotic events raises the risk of broad application of this drug (Kuenen et al 2002).

B. *Tyrosine Kinase/EGFR Inhibitors*:

1. **ZD 1839 ('Iressa')** *main features and Action Mode:*

a. **EGFR**= Epidermal Growth Factor Receptor *activates* several *downstream* signaling pathways and bis overexpressed in numerous types of human cancers, including: non-small cell lung (NSCLC), colorectal, head and neck, bladder, brain, pancreas, breast, ovary, prostate, and gastric cancers (Salomon et al 1995; Gullick et al 1991). Overexpression of EGFR is associated with increased invasiveness, resistance to treatment and poor outcomes in several tumor types (Neal et al 1985; Ke et al 1998);

b. Found to be effective in the treatment of: *Non-small cell lung (NSCLC), colorectal, head and neck, bladder, brain, pancreas, breast, ovarian, prostate and gastric **cancer types that were previously unresponsive to other chemotherapy*** (Salomon et al 1995; Gullick et al 1991*)*;

c. ZD 1839 (Iressa) blocks EGFR; ZD1839 inhibits autophosphorylation, and resulted in complete regression in some xenograft tumors (Ciardiello et al 2000; Sirotnak et al 2000) when used with cytotoxic drugs such as doxorubicin, or in combination with radiation;

d. Iressa inhibits the Ras/MAP kinase and STAT-3 transcription factors**,** in many tumors**;** the inhibition of the epidermal growth factor receptor (EGFR) has been of significant interest lately, partially because of the autocrine activation of EGFR and several downstream pathways, such as the ras/MAP kinase and STAT-3 transcription factors, in several tumors. The

activated EGFR pathway *induces entry into the cell cycle, inhibition of apoptosis, and also activation of angiogenesis and motility*. Several phase I and II studies with Iressa have already been completed (Ferry et al 2000; Negoro et al 2001; Baselga et al 2000). Daily oral doses have ranged from 50 to 700 mg for 2 to 4 weeks. ZD1839 resulted in some responses in NSCLC and prostate cancer, and stability of disease (over 4 months) in several patients (Ferry et al 2000; Negoro et al 2001; Baselga et al 2000). 22% of Japanese patients achieved partial response (Negoro et al 2001). Side effects have been relatively mild and have included diarrhea and rash.

2. **OSI-774** *or Erlotinib, or 'Tarceva'*:

‘Tarceva’ is also an EGFR inhibitor; it binds very tightly to EGFR, causing EGFR inhibition, and also produces downstream inhibition of the P13/MAPK signal transduction pathways, *resulting in accumulation of p27, that leads to cell cycle arrest at the G1 phase and induction of apoptosis* (Moyer et al 1997). EGFR-TK is more than 1000 fold sensitive to ‘Tarceva’ compared with any other tyrosine kinases. Therefore, it is a very specific inhibitor of EGFR –TK and *reduces very markedly the phosphorylated EGFR-TK*;

a. The IC_{50} for ‘Tarceva’ is 2 nM (when measured by purified EGFR-TK inhibition in biochemical assays), and its value is 20 nM for the EGFR-TK autophosphorylation when measured in intact cells;
b. Proposed mechanism of action: reversible inhibition of EGFR-TK through competitive binding to the ATP site;
c. Results of preliminary Clinical Trials: partial responses in patients with colorectal cancer and renal cell carcinoma (kidney), as well as > 5 month stabilization in: colon, prostate, cervical, NSCLC and head and neck cancers.

3. *‘Herceptin’*

a. Trastuzumab, a recombinant humanized monoclonal antibody directed against HER2 is known as ‘Herceptin’ (Carter et al 1992). The HER2/neu gene increases the kinase activity, initiating signal transduction, leading to proliferation and differentiation in approximately 30% of human breast cancers (up to 50 to 100 gene copies/cell);
b. The HER2/neu gene makes a type I receptor tyrosine kinase encoding a 185 kDa surface membrane receptor protein;
c. Phase I trials showed that the dose of trastuzumab (i.v. 10 to 500 mg single dose or weekly) could be increased without toxicity and that pharmacokinetics were dose-dependent (Shak et al 1999). Phase II trials response is > 5.3 months. Phase III trial patients received doxorubicin or epirubicin plus cyclophosphamide, and 28% of patients treated with chemotherapy and trastuzumab were free of tumor progression, compared with 9% of the patients treated with chemotherapy alone.
d. The monoclonal antibody of the membrane receptor HER2 signaling protein is much more efficient than chemotherapy alone. About 1 in 5 of the patients had cardiac dysfunction, where trastuzumab was at 4 mg/kg body weight initially.
e. A phase II trial was conducted with 46 HER2 (+) metastatic breast cancer patients who had failed prior cytotoxic chemotherapy (Baselga et al 1996). Objective responses were seen in 5 of 43 assessable patients, including 1 complete remission and 4 partial remissions. A second phase II trial (Pegram et al 1998) combined

trastuzumab with cisplatin in 39 HER2 (+) metastatic patients who had failed prior chemotherapy. Of the 37 subjects, 9 achieved a partial response and 9 had a minor response or stability. A randomized, placebo-controlled phase III study was performed to determine efficacy and safety of adding trastuzumab to chemotherapy in breast carcinoma. 28% of patients treated with both were disease progression-free at 12 months, compared with 9% of the patients treated only with 'standard' chemotherapy.

f.   The treatment is indicated as a single agent for patients that have failed earlier therapy, and it is used also as first-line treatment for metastatic disease when used in combination with paclitaxel.

g. Trustuzumab *has already been approved by the FDA for use in women with metastatic breast cancer with HER2-positive tumors*.

4. Cetuximab

h.   An antibody-based approach to affecting tyrosine kinase signaling is by cetuximab, a humanized monoclonal antibody against the EGFR. MAb225, a murine monoclonal antibody that specifically binds to EGFR, specifically competes with signal transduction initiated by TGF-α (Gill et al 1984). Cetuximab (C225) is the human-mouse chimeric version of Mab225, which specifically binds to the EGFR with high affinity, preventing the ligand from interacting with the receptor.
i.   Preclinical studies show that cetuximab results in cell-cycle arrest as well as apoptosis in different contexts (Huang et al 1999; Peng et al 1996). A synergistic effect of cetuximab with cytotoxic chemotherapy has been seen with cisplatin, doxorubicin (Baselga et al 1993), gemcitabine (Bruns et al 2000), docetaxel (Tortora et al 1999), and paclitaxel (Inoue et al 2000).
j.   Early phase I trials demonstrated that cetuximab displays nonlinear, dose-dependent pharmacokinetics that are not altered by coadministration of cisplatin (Baselga et al 2000). These studies were conducted in patients with tumors overexpressing EGFR. There were only 5 episodes of severe C225-related toxicities among the 52 patients. Two patients with head and neck tumors who received cetuximab at doses of 200 mg/m^2 and 400 mg/m^2 with cisplatin exhibited a partial response. In light of these results, the clinical development of cetuximab is continuing with a number of phase II and III studies.

Serine-Threonine Kinase Antagonists (Stkas)

1. Rapamycin Congeners:

a.   Rapamycin (Sirolimus, Rapamune) is a macrolide fungicide that binds intracellularly to the immunophilin FKBP12, and the resultant complex inhibits the activity of a 290-kDa kinase known as mammalian target of rapamycin (mTOR). Rapamycin is isolated from the bacteria *Streptomyces hygroscopicus* and is found to have potent antimicrobial and immunosuppressive properties (Baker et al 1978).

b.   Sirolimus *was approved by the FDA for prevention of allograft rejection after organ transplantation* (Sehgal et al 1995).

c.   Further studies with rapamycin revealed significant antitumor activity (Eng et al 1984). This is understandable given the importance of mTOR in mitogenic cell signaling. mTOR is a kinase member of PI3K-related kinase family that is activated in response to growth signaling through the PI3K/Akt pathway. Activation of mTOR results in increased translation of several critical cell-cycle regulatory mRNAs through two downstream effector kinases, p70S6K and 4E-BP1/PHAS (Sekulic et al 2000; Gingras et al 1998). Rapamycin causes G_1 cell-cycle arrest by increasing the turnover of cyclin D1 (Hashemolhosseini et al 1998), preventing upregulation of cyclins D3 and E (Decker et al 2001), upregulating p27^{KIP1}, and inhibiting cyclin A-dependent kinase activity (Kawamata et al 1998). Several analogs of rapamycin have been selected for further development as anticancer agents.

d.   CCI-779, an ester of rapamycin, has significant antiproliferative effect and favorable toxicology profile and is being studied in several phase I trials in humans (Hidalgo et al 2000; Raymond et al 2000). Several partial responses have been documented in renal cell carcinoma, NSCLC, neuroendocrine tumors, and breast cancer, in addition to minor responses or stable disease in several tumor types (Hidalgo et al 2000; Raymond et al 2000). RAD001, an orally bioavailable hydroxyethyl ether derivative of rapamycin, also has potent activity against various animal xenograft models of human tumors; an antiangiogenic effect may account in part for its antiproliferative properties (O'Reilly et al 2002).

2. MEK Inhibitor PD 184352:

e.   The stimulation of *Ras-mediated signal pathways* results in a cascade of downstream kinase activation including Raf, which phosphorylates two distinct serine residues on the dual-specificity kinase MEK (MAP kinase-kinase) (Lewis et al 1998). MEK, in turn, activates and exclusively phosphorylates two subsequent kinases, ERK1 and ERK2 (MAPK), on specific tyrosine and threonine residues within each kinase. These kinases phosphorylate a variety of substrates including transcription factors critical to cell proliferation and tumor invasion (e.g., Marais et al 1993).

f.   In cytotoxicity studies, correlation between sensitivity to PD184352 and increased activated MAPK levels was observed in some cells—in particular, colon cancer cells. Higher levels of MAPK activation were observed in colon tumor tissue versus normal mucosa as this event occurs late in colon carcinogenesis (Sebolt-Leopold et al 1999).

g.   In mice with colon 26 xenograft model treated with PD184352, excision and assay of tumor cells revealed diminished phospho-MAPK levels. After drug withdrawal, a return to baseline levels was observed reflecting the cytostatic nature of the inhibition. The pharmacodynamic measurement of activated MAPK in tumor tissue may be used as a biological marker of drug activity as antibodies specific for phosphorylated MAPK are routinely available.

3.   Bryostatins:

a.   The bryostatins represent a large family of secondary metabolites produced in extremely small amounts by the marine invertebrate, *Bugula neritina* of the phylum *Ectoprocta* (Pettit et al 1991). The various bryostatins are distinguished by varying side chains off the macrocyclic lactone ring structure. Despite this close structural relationship, these nontumor-promoting PKC activators have different biologic

activities and spectrum of toxicity (Kraft et al 1996; Jones et al 1990). Bryostatin 1 (Bryo 1) is the prototype of this 17-member family and the most extensively studied in humans. Initial isolation of Bryo 1 was based on its antineoplastic activity against the murine P388 lymphocytic leukemia. Bryo 1 is a potent and rapid activator of PKC; however, unlike other PKC activators, including phorbol myristate acetate (PMA), Bryo 1 lacks tumor-promoting capabilities.

b.   The first two published phase I trials evaluated Bryo 1 administered as a 1 h intravenous infusion (Prendiville et al 1993; Philip et al 1993). The DLT was myalgia, occurring approximately 48 h after treatment and lasting up to several weeks at the highest dose levels (65 μg/m^2/dose). The MTD was 50 μg/m^2, and the recommended dose for phase II trials was 35 to 50 μg/m^2 every two weeks. Partial responses were observed in two patients with malignant melanoma, which lasted 6 months and 10 months. Plasma levels of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) increased 2 h and 24 h after treatment, respectively, and were dose related.

4. *UCN-01* (**7-OH** *Staurosporine*):

c.   Staurosporine, a natural product isolated from *Streptomyces staurosporeus*, is a relatively broad, nonspecific protein kinase antagonist, originally isolated in an effort to define inhibitors of protein kinase C (PKC). 7-OH staurosporine (UCN-01) was defined as a more selective, but not specific, PKC antagonist.

d.   Two prominent effects of UCN-01 have emerged in preclinical studies in vitro: induction of cell-cycle arrest, and abrogation of the checkpoint to cell-cycle progression induced by DNA damaging agents. UCN-01 inhibited cell growth in several in vitro and in vivo human tumor preclinical models (Akinaga et al 2000); however, antiproliferative activity on the part of UCN-01 cannot be explained solely by inhibition of PKC. First, in cell-cycle analyses UCN-01 *inhibits Rb$^+$* cells at G1/S phase of the cell cycle (Akiyama et al 1997). In addition, cells treated with various concentrations of UCN-01 showed *decreased pRb phosphorylation in a dose-dependent manner* (Chen et al 1999). These results suggest that CDK2- or CDK4-regulated steps are targets for UCN-01-induced cell-cycle arrest.

e.   UCN-01 abrogates the DNA damage-induced checkpoints to cell-cycle progression in G2 (Bunch et al 1996; Wang et al 1996) and in S phase (Shao et al 1997). It is noteworthy that these effects were apparent at drug concentrations that appeared to have little direct effect on cell proliferation or that caused enhanced cytotoxicity by clonogenic or proliferation assays. In addition, they provided a mechanistic framework for prior observations that DNA-damaging agents such as mitomycin (Akinaga et al 1993) could greatly potentiate UCN-01 action.

f.   In contrast to animal studies, UCN-01 displayed strong binding to human plasma proteins, apparently to the α1-acid glycoprotein (AAG) in initial human phase I clinical trials (Sausville et al 2001; Fuse et al 1998). One partial response occurred in a patient with melanoma, and a protracted (>4 year) period of stabilization of minimal residual disease was observed in a patient with *alk*(+) anaplastic large cell lymphoma.

D. *Miltefosine and Perifosine (ALP Analogs):*

g.　　Certain alkylphospholipids (ALP) (e.g., Rac-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine [ET-18-OCH$_3$, edelfosine]) when given to mice prior to transplantation of Ehrlich ascites carcinoma cells, effectively prevent growth of this tumor (Tarnowski et al 1978). Enhancement of immune defense against tumor cells was initially considered a plausible mechanism and has been demonstrated on multiple occasions by a number of ALP analogs.

h.　　Edelfosine is also able to induce apoptosis in HL60 leukemic cells, even in low concentrations and after short incubation times. In U937 leukemic cells, the compound induced apoptosis rapidly, whereas in epithelial HeLa tumor cells this induction required prolonged times of treatment (Mollinedo et al 1993).

i.　　All ALP analogs studied so far cause an indirect inhibition of PKC, most likely as a result of the reduced formation of diacylglycerol through inhibition of phospholipase C (Uberall et al 1991; Seewald et al 1990). Additional antiproliferative mechanisms could involve altered growth factor receptor function, as well as recent evidence of *p21 induction by an as yet undefined pathway* (Patel et al 2002) *irrespective of p53 function.*

j.　　Eight phase I-II studies, consisting of 443 patients using topically applied miltefosine 2%-8% for skin metastases in patients with breast cancer, showed a median response rate of 38% (Unger et al 1988; Smorenburg et al 2000; Terwogt et al 1999). Evidence from the trials led to the approval of miltefosine, licensed as Miltex$^©$, in Germany for the treatment of cutaneous breast cancer and cutaneous lymphomas.

k.　　The heterocyclic alkylphosphocholine derivative octadecyl-(1,1-dimethyl-piperidino-4-yl) phosphate (D-21266; perifosine) was developed and selected for improved gastrointestinal tolerability. A number of phase I studies are presently ongoing in Europe and the United States; early evidence points to better tolerability and less gastrointestinal toxicity (Messmann et al 2001).

E. *Proteasome Inhibitor PS-341:*

l.　　The proteasome, a multicatalytic protease responsible for degradation of most proteins with the cell, has emerged as a new target for anticancer drug development. The 20S proteasome is involved in the degradation of several cell-cycle regulatory proteins such as cyclins (A, B, D, E), cyclin-dependent kinase inhibitors (**p21**[WAF1/CIP1] and **p27**), oncogenes (c-fos/c-jun, c-myc, N-myc), and p53 and regulatory proteins (IκB, p130) (King et al 1996). Inhibition of the 20S proteasome pathway, therefore, aims at altering the cell cycle to promote apoptosis (An et al 2000). Although the proteasome is present in all cells, transformed and dividing cells are most sensitive to its inhibition (Drexler et al 1997).

m.　　PS-341 is the first proteasome inhibitor to enter human trials. It is a boronic acid dipeptide that specifically inhibits the 20S proteasome presumably through the stability of a boron-threonine bond that forms at the active site of the proteasome. It was found to have substantial cytotoxicity against a wide range of human tumor cells

in the NCI 60 cell line anticancer drug screen (Adams te al., 1999). PS-341 causes accumulation of cyclin A, cyclin B, **p21$^{WAF1/CIP1}$**, and wild-type p53 and arrests the cells at the S and G$_2$/M phases followed by nuclear fragmentation and apoptosis. PS-341 significantly inhibited NF-κB DNA binding and functional reporter activity (Sunwoo et al 2001). Several phase I studies evaluated various schedules of PS-341 administration. At the MTD recommended for phase II studies (1.25 mg/m^2-1.3 mg/m^2), a 65%-72% inhibition of 20S proteasome was achieved (Erlichman et al 2001; Aghajanian et al 2001). An average 54% inhibition of proteasome was achieved in patients' tumors (Hamilton et al 2001). In these phase I studies several patients achieved partial responses and disease stabilization including a bronchoalveolar NSCLC, melanoma, sarcoma, lung adenocarcinoma, and malignant fibrous histiocytoma. Patients usually had more toxicity with the second cycle of treatment. Currently several phase II clinical trials are evaluating PS-341 as a single agent in hematologic malignancies, neuroendocrine, renal cell, melanoma, breast, brain, pediatric tumors, and several other solid tumors. Significant antitumor effects were documented in a phase II study of PS-341 in refractory multiple myeloma (Richardson et al 2001).

F. *Farnesyl Transferase Inhibitors*:

a. Ras genes are mutated in 30% of all human cancers with K-Ras being the most common. This family of genes encodes GTP binding *proteins that are important in malignant transformation, cell growth, and intracellular signal transduction.*

b.	Normal ras binds GTP and in the GTP-bound state interacts with numerous effectors including the *raf* proto-oncogene kinase and phosphatidyl-inositol 3-kinase.

c.	Three isoforms, Harvey (Ha), Kirsten(K), and N-isoforms have been described, with mutation of the GTPase of the K isoform resulting in a persisting signaling capacity in approximately 20% of human epithelial tumors. N-ras is mutated in a smaller proportion of malignancies, predominantly leukemias. Ras function requires lipophilic anchorage to the cell membrane by lipid prenylation. This requires posttranslational modification or covalent thioether bond formation between a farnesyl group (C15) and a cysteine residue at the ras carboxy terminus. A "GTT shunt pathway" maintains K-Ras in an active prenylated, membrane-bound form and explains in part the requirements for higher farnasyl transferase inhibitor (FTI) dose or co-treatment with a GTT inhibitor for significant growth inhibition in K-Ras models (Pendergast et al 2000). Several classes of FTIs have been developed in an initial effort to define inhibitors of Ras function and, in general, compete with the enzyme substrates, the CAAX tetrapeptide, and farnesyl pyrophosphate (FFP). The CAAX competitors are generally peptidomimetic agents that mimic the carboxyl terminal portion of the Ras protein.

1. *SCH66336*:

a.	SCH66336 is a novel oral agent derived from a class of nonpeptide, nonthiol-containing, CAAX mimetic FTIs (Bishop et al 1995). The drug inhibits in vitro FT activity with an IC$_{50}$ of 1.9 nM for H-ras, 2.8 nM for N-ras, and 5.2 nM for K-ras. Inhibition of cells with activated ras and anchorage-independent growth was noted with IC$_{50}$ 75 nM in H-ras versus 400 nM with K-ras-driven cells (Liu et al 1999). The observed growth inhibition of tumor cells in soft agar and in xenografts was

independent of ras mutational status because even wild-type ras cells were sensitive (Liu et al 1998).

b. The phase I experience with SCH66336 involved 20 patients using a twice a day schedule over 7 days every 21 days. Eight patients had stable disease, and treatment for up to 10 cycles was possible in a few patients. Antitumor activity was reported in only one patient with advanced NSCLC who had a greater than 50% reduction in an adrenal metastasis and received treatment for 14 months (Adjei et al 2000); further studies will be needed as statistical significance has not been achieved.

2. *R115777:*

R115777 is a substituted quinolone and competitive inhibitor of the CAAX peptide binding site of FT (End et al 1999). The compound inhibits in vitro K-Ras farnesylation (IC_{50} 7.9 nM) and exerts antiproliferative effects in cell lines such as H-Ras-transformed fibroblasts (IC_{50} 1.7 nM) and K-Ras-driven colon and pancreatic cells lines (at roughly IC_{50} 20 nM) (End et al 2001).

a. The initial clinical experience with R115777 in 27 patients was reported by Zujewski et al. (2000). A patient with metastatic colon cancer had symptomatic improvement and a 50% reduction in carcinoembryonic antigen (CEA) levels.

b. A most interesting outcome was obtained in patients with myelodysplastic syndrome or relapsed or poor prognosis leukemias, where a phase I dose escalation study revealed DLT at 1200 mg twice per day, consisting of neurotoxicity, with non-DLTs including renal insufficiency and myelosuppression. There was clear evidence of downmodulation of erk kinase activity, along with the farnesylation status of lamin A and HDJ-2. Clinical responses occurred in 29% of 34 evaluable patients, including 2 complete responses (Karp et al 2001). Though there were no mutations in N-Ras detected in this patient population, this study did suggest that in addition to clinical activity there was some evidence of downmodulation of signaling as well as farnesylation-directed activities.

The results summarized in this section convey the promise, as well as some of the challenges, encountered in developing signal transduction inhibitors for cancer treatment. These molecules represent a distinct departure from previous therapeutic approaches based on cytotoxic activity in tumor models, without reference to underlying mechanism. The fact that any responses have been seen at all reaffirms the relevance of tumor cell biology in charting the further course of cancer developmental therapeutics. However, the initial experiences raise a number of issues that should be considered before the field advances.

First of all, with certain agents the actual magnitude of conventionally described responses is lower than would usually be associated with clinical value. A more accurate means of diagnosing the dependence of a tumor on a particular signaling pathway or target must be defined. Microarray, proteomic and interactomic approaches offer this promise, but these must be integrated into the clinical trials process. Some agents have entered initial clinical trials with extensive efforts to document target-based effects in conjunction with pharmacology and clinical toxicity evaluations; other agents have not, however, and in those instances the phase I study lacks depth as far as valuable information is concerned. Lacking clear evidence of clinical response, one cannot confidently move forward to the next phase.

Intelligent design of combinations with standard cytotoxic agents also remains a challenge. Preclinical models of synergistic effect with signaling agents often proceed from empiricism without understanding a mechanistic basis that would guide clinical implementation. These circumstances call for renewed efforts to define better assays of target effect in the preclinical phase of a drug's development that can be translated to the clinical arena.

Many of the agents in both model systems and in initial clinical observations in patients might be associated with protracted periods of disease stability, rather than overt cytotoxic effects that might be attributed to the initiation of an apoptotic response. Though such stable disease can be readily observed in animal tumor models, it is uncertain whether it can be meaningfully captured in clinical populations of patients with advanced cancer. We must develop decision-making steps that will aid in the use of the drugs in patients at earlier cancer stages or indeed adjuvant/prevention strategies, and to develop clinical study algorithms that address this biologically relevant possibility in a way that does not compromise patient safety.

Despite these issues, it is clear that this generation of molecules has marked a turning point in cancer therapeutics and defined a path for future progress. We look forward to the day when cytotoxic strategies will be fully employed and in coordination with rationally based signaling strategies that address the molecular disorder of cancer as a basis that will benefit the cancer patients.

There is clearly a need for individualized cancer therapy strategies based on high throughput micoarray information recorded for isolated tumor cell lines from stage I through stage III cancer patients; such data is essential for improving the survival rate of stage III cancer patients undergoing clinical trials with novel signaling pathway modulator or blocker medicines, such as those discussed in this section. Specific methodologies and suggestions for developing such strategies for personalized cancer treatments will be presented in the next subsections.

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Additional Notes on Signaling Payhways Inhibitors:

N1. We need to test through quantitative modeling the combined effects of cytostatic anticancer drugs combined in different proportions with the new signaling-based anticancer complement drugs.

N2. The second drug of this type is Cetuximab (C225) that is going to Phase II and III clinical trials.

N3. Preclinical studies with C225 showed that Cetuximab results in cell cycle arrest, as well as apoptosis in several types of tumors, and it had synergistic effects with cytotoxic chemotherapy.

N4. *Regarding Flavopiridol*:

a.   Flavopiridol causes cell-cycle arrest at G_1/S phase transition and G_2/M phase transitions and also slows the progression of the cell cycle through the S phase (Kaur et al 1992).
b.   The goal is to develop new STKAs that would be similar to flavopyridol, or HMR 1275;

c.   Some STKAs act as blockers of Cyclin D1, therefore causing cycle arrest by direct transcription repression of cyclin D1 mRNA (79), and in mantle cell lymphoma, flavopyridol delayed significantly progression of disease in 84% of patients (80);

d.   Cytostatic effects are significant and were observed with the flavopyridol in colorectal and prostate carcinoma xenograft models (70, 81);

e.   These findings prompted an evaluation of cyclin-dependent kinases, recognized as responsible for governing the orderly transition from G_2 to M phase (CDK1) and G_1 to S phase (CDK4 or 6 with CDK2). Indeed, flavopiridol inhibits all CDKs known so far (IC_{50} ∼100 nM), inhibiting CDK1, CDK2, and CDK4 with a similar potency (Carlson et al 1996).

f.   Results: Risk of thrombosis (among others). Preclinical studies of flavopiridol revealed wide differences in growth inhibition between cell types depending on the duration of exposure and concentration of the drug.

g.   Significant cytostasis is observed when flavopiridol is administered in protracted fashion to colorectal (colo 205) and prostate (LnCap/DU145) carcinoma xenograft models (Senderowicz et al 2000; Drees et al 1997). Shorter "bolus" administration of flavopiridol to a lymphoma/ leukemia (HL60) cell line had a higher degree of apoptosis and cytotoxicity (Arguello et al 1998).

h.   Some cases of partial responses and stable disease have been reported in various phase I studies, but several phase II studies revealed few conventionally defined responses in several tumor types, with the possible exception of mantle cell lymphoma (Connors et al 2001; Schwartz et al 2001; Shapiro et al 2001; Stadler et al 2000). In the future, Flavopiridol may be used in combination with other agents, including taxol (Motwani et al 1999), irinotecan (Motwani et al 2001), and gemcitabine (Jung et al 2001), as well as other signal transduction modulators (Yu et al 2001; Nguyen et al 2002; Yu et al 2002).

5.3. Interactome-Transcriptome Analysis and Differential Gene Expression in Cancer

It has been claimed that high-throughput yeast-two-hybrid (HT-Y2H) methods will allow a systematic approach to functional genomics, by placing individual genes in the global context of cellular functions (Mendelsohn and Brent, 1999). One finds that high-throughput screening methods such as HT-Y2H have indeed allowed the mapping of the first interactomes for three eukaryotes (Giot et al 2003; Li et al 2004; Uetz et al 2000). Because of the human interactome's much larger size and its very high-degree of complexity there will be quite high costs and labor involved in obtaining the data necessary, for example, for an HT-Y2H mapping of a complete human cell interactome. Furthermore, the complete data analysis together with the assembly of the complete interactome network is likely to require both conceptual and computational advances, in addition to a significant amount of time and collective effort(s) by one or several research teams. In view of the high, potential importance of the human interactome for cancer therapy, and also for improved diagnosis and 'rational' clinical trials, such an effort *should now be the top priority* or, at the very least, must be given a priority far above that of 'simpler' projects for the smaller-sized interactomes. Such an effort should also be coordinated with an improved mapping of the complete yeast interactome as a model, or test, system. Meantime, there have been since 2005 a few reports of 'surrogate', or partial, human cancer cell interactomes in the form of predicted maps of human protein interaction networks based on partial data and comparative analysis. Such studies emphasize even further the need and urgency for the complete mapping of several human cancer cell interactomes.

Following the seminal studies of DeRisi et al (1996) that utilized cDNA microarray to analize

gene expression patterns in human cancer, there have been relatively few attempts at deriving hypothetical gene expression patterns in human cancer. The first claim of such an attempt was recently made by Wachi, Yoneda and Wu (2005) for genes that were differentially expressed in squamous cell lung cancer tissues from five patients who had undergone surgical removal of the tumor(s). cRNA samples were prepared and hybridized to arrays obtained from Affymetrix® (Hg-U133A^{TM}.). These authors were able to carry out paired *t*-test analyses for each *individual* patient in order to distinguish the genes in which expression levels in their squamous lung cancer cells differed from the paired normal lung tissue (control samples) obtained from the same five individuals. The authors' prediction methodology will be briefly discussed in the next subsection as some of the details are relevant for the evaluation of these results which were the first to be reported for the (hypothetical) interactome—transcriptome analysis of human cancer cell data for a group of five patients with the same diagnosed form of (lung) cancer, and with the same treatment (tumor removal by surgery).

The hypothetical human protein interaction maps are a relatively new endeavor (Brown and Jurisica, 2005; Lehner and Fraser, 2004) perhaps because they are likely to have many false positives, as well as miss a significant fraction of the relevant/real protein-protein interactions. Currently, microarray analysis still suffers inherently from relatively high noise levels and the accompanying information loss (buried in noise); although this inherent noise problem is partially eliminated through multiple replicate analyses, the number of replicates is often limited by the availability and the material cost. Another significant problem of such microarray projects is the huge amount of data that needs to be processed in order to obtain useable information (Claverie, 1999).

5.3.1. Analysis of Human Protein-Protein Interactions (HPPI) and Integration of array data into a Predicted Protein-Protein Interaction Network (PPIN), (summarized from Wachi, Yoneda and Wu, 2005).

Wachi, Yoneda and Wu (2005; WYU05) employed for their human cell data analysis a web-presented database (OPHID, April 25, 2005) of predicted interactions between human proteins (Brown and Jurisica, 2005) based on data for human and other four organisms which included the intensely-studied yeast and fruit fly. (OPHID is freely available to academic users at http://ophid.utoronto.ca). This protein interaction database listed 16,034 known human protein interactions obtained from various public protein interaction databases, as well as 23,889 additional, predicted interactions which are evaluated using protein domains, gene co-expression and Gene Ontology terms. The results can be visualized in OPHID using a customized, graph visualization program. The data comprises literature-derived human PPI from BIND, HPRD and MINT, "with predictions made from *Saccharomyces cerevisiae, Caenorhabditis elegans, Drosophila melanogaster* and *Mus musculus"*. The genes in the WYU05 array were matched to those in OPHID using gene symbols and protein sequences. In this manner, 2137 genes in the WYU05 microarray experiments were 'matched to the protein network from OPHID'. These predictions should, however, be thought of only as 'hypotheses' until they are experimentally validated. On the other hand, there is increasing evidence that at least certain PPIs may be conserved through evolution (Pagel et al 2004; Wuchty et al 2003). Recently, Sharan et al (2005) claimed that about 50% of the protein-protein interactions predicted by using interologs between microorganisms are also experimentally validated. The interologs approach might play therefore a role in the partial validation of the HT-Y2H protein network mapping without, however, necessarily achieving the claimed, global validation of the predicted (hypothetical) interactome.

Differentially expressed genes (DEGs) from squamous cell carcinomas (SCCs) were then identified as discussed above and their connectivity in the network graph was examined to determine their 'topological' properties, such as the edge distribution for DEGs in comparison with the surrounding graph subnetwork.

5.3.2. Differentially expressed genes –*DEG- Results for SCC of Human Lung*
(*summarized from Wachi, Yoneda and Wu, 2005*).

The genes that are upregulated in SCC were found to exhibit a positive correlation (Pearson's r-coefficient of 0.82) with the number of edges associated with them (Fig. 1a of Wachi, Yoneda and Wu 2005), which was interpreted as indicating that DEGs that are upregulated in SCC are also highly connected. However, the downregulated genes were reported also to have a positive correlation (r = 0.75) to connectivity, albeit slightly lower (Fig. 1b of Wachi, Yoneda and Wu, 2005). On the other hand, microarray probesets that matched the genes in the protein network (n =-2,137) had a negligible correlation coefficient (r =0.06) to link number, proving that the genes on the test microarrays did not contribute to bias in the number of links for DEGs in SCC.

A k-core analysis of DEGs in SCC of the human lung was also carried out *(loc. cit*.) which were reported to measure "how close are the DEGs to the topological 'center' of the human PPI network". Based on the k-core analysis, it was concluded that: "*the upregulated genes are more centrally located in the protein network than the downregulated genes*". If duplicated and validated, such studies would be important as the 'topological centrality' of the genes in the interactome was previously reported to be associated with the *essential* functions of the genes in the yeast (Jeong et al 2001). Such essential genes, are lethal when mutated, and also tend to have high connectivity. Moreover, other genes that are not essential in this sense, but provide a vital function in toxin metabolism were reported to have a high number of edges associated with the nodes, and to be less well connected than the essential genes in yeast (Said et al 2004). Furthermore, a *k*-core analysis has also been performed on the yeast essential genes and they were reported to be global hubs, whereas the non-essential genes were not hubs (Wuchty and Almaas, 2005). It was also claimed that these essential, global hubs are conserved throughout different species; however, one notes that, thus far, there is insufficient data and evidence to prove this claim, or hypothesis. Nevertheless, one may consider as a 'working hypothesis' that "*there should be a core set of genes that needs to be maintained throughout the course of somatic evolution in the tumor microenvironment*" (Wachi, Yoneda and Wu, 2005). This hypothesis is thus consistent with the *somatic evolution model* of cancer. Such conserved genes might be the 'essential genes' in cancer cells, and they may also have somewhat analogous to the global hub, essential genes reported in yeast (Wuchty and Almaas, 2005). DEGs would thus be essential for the survival and proliferation of cancer cells in SSC of the human lung, and the upregulated genes would be centrally located in the protein network as well as have higher connectivity, perhaps suggesting their possible essential role(s) in human (SSC) lung cancer. As this is the first report of a predicted/ hypothetical human cancer interactome network one should definitely consider 'replicating' the reported studies and also evaluating such potentially important findings in the context of a complete human cancer interactome (differential) analysis. This possibility that DEGs might be essential for the survival and proliferation of cancer cells in SSC of the human lung has much too important consequences to be ignored; therefore, it must be thoroughly investigated and also tested with sufficiently extensive, translational genomics and transcriptional databases that do not seem to be currently available (Han et al. 2006). Further supporting

analyses for this conjecture made by Wachi, Yoneda and Wu (2005) are considered in the next section.

5.4. *Cancer Proteins and the Global Topology of the Human Interactome Network*

A recent and extensive study of both cancer and non-cancer proteins (Jonsson and Bates, 2006) was integrated into a validated protein-protein interaction (PPI) network, or interactome, of human proteins. In their report, the connectivity properties were investigated for all proteins previously shown to be modified as a result of mutations leading to cancer (Furteal, et al 2004). A global protein-protein interaction network was then constructed by a homology--based method which is claimed to accurately predict protein-protein interactions. It was then suggested that human proteins that are involved in cancer, or 'cancer proteins', exhibit a network topology which is substantially different from that of other proteins which are considered not to be involved in cancer. Notably, increased connectivity was pointed out for cancer proteins involved in the following subnetworks: cell growth and apotosis-related, signal transduction (MAPK, TGF-beta, insulin, T-cell and B-cell receptor, adipocytokine, cytokine-cytokine interaction), cell motility/cytoskeleton, cell communication, adherence junction, focal adhesion, leukocyte migration, antigen processing and folding/sorting/degradation. Furthermore, it was proposed that such observations '*indicate an underlying evolutionary pressure to which cancer genes, as genes of central importance, are subjected.'* Linking these claims with previous proposals by Wuchty and Almaas (2005) that globally central proteins form an evolutionary backbone of the proteome and are *essential* to the organism, (and also with the conjecture made by Wachi, Yoneda and Wu, 2005, discussed here in Section 5.3.), Jonsson and Bates (2006) suggested that cancer proteins may generally be older than the non-cancer ones in evolutionary age. Furthermore, they also suggested that the somatically mutated cancer proteins may be of somewhat younger evolutionary average age in comparison with those from the germline, as a consequence of the evolutionary selection pressure postulated to affect germline mutated proteins. Note also that the previous study of (SCC) human lung cancer by Wachi, Yoneda and Wu (2005) also reported increased interaction connectivity in differentially expressed proteins in human lung cancer tissues.

6. Epigenomics in Mammalian cells and Multi-cellular Organisms

6.1. *Epigenetic Controls*

Upon completion of the US Human Genome Mapping Project and related studies, it became increasingly evident that a sequence of 30,000 or so 'active' genes that encode and direct the biosynthesis of specific proteins could not possibly exhaust the control mechanisms present in either normal or abnormal cells (such as, for example, cancer cells). This is even more obvious in the case of developing embryos or regenerating organs. Subsequently, more than 120,000 genes were suggested to be active in the human genome (*Nature, 2004*). Furthermore, specific control mechanisms of cellular phenotypes and processes were recently proposed that involve *epigenetic* controls, such as the specific acetylation <—> de-acetylation reactions of DNA-bound histones (for an overview article on epigenomics see, for example, *Scientific American* 2003, December issue). Such controls intervene from outside the genome but ultimately they also affect gene expression. Therefore, gene profiling techniques would need to be combined with epigenomic tools and analysis in order to gain an improved understanding of functional genomics and interactomics. Epigenomic tools and novel techniques begin to address the complex and varied

needs of epigenetic studies, as well as their applications to controlling cell division and growth. Such tools are, therefore, potentially very important in medical areas such as cancer research and therapy, as well as for improving 'domestic' animal phenotypes *without* involving genomic modifications of the organism. This raises the interesting question if 'epigenomically controlled-growth organisms' (ECGOs) -- to be produced in the future-- would be still argued against by the same group of people who currently objects to GMOs, even though genetic modifications would be neither present nor traceable in such ECGO organisms?'

6.2. Novel tools in Epigenomics: Rapid and Ultra-sensitive Analyses of Nucleic acid –Protein Interactions

Several novel techniques could also be applied for the highly-selective detection of epigenomic changes in mammalian cells related to diseases such as individual types of cancer (Jones and Laird, 1999; Plass, 2002) and Alzheimer disease. Such novel tools are likely to be utilized in a wide range of applications in biotechnology research related to Post-Genomics and Epigenomics. Tumor suppressor genes are transcriptionally silenced by promoter hypermethylation that also appears to lead to alterations in chromatin structure- a possible mechanism for such repression of the suppressor genes. In contrast to the genetic mutation or deletion mechanism of tumor suppressor gene inactivation, epigenetic inactivation of tumor suppressor genes would occur *via* methylation of specific DNA regions that could be prevented by DNA methyl-transferase or histone deacetylase inhibitors. Aberrant CpG--island methylation has non-random/tumor-type-specific patterns (Costello et al 2000). Such patterns can be identified by employing methylation--specific PCR (MS-PCR; Herman et al 1996), and can also be employed either for tumor class prediction by microarray-based DNA methylation analysis (Adorjan et al 2002) or for high-throughput microarray-based detection and analysis of methylated CpG islands (Yan et al 2002). Hypermethylation profiling is important for both accurate diagnosis and the development of optimal strategies in cancer therapy. Gene promoter hypermethylation has been reported in both tumors and serum of patients diagnosed with several types of cancer: head and neck cancers (Sanchez-Caspedes et al. 2000), nasopharyngeal carcinoma (Wong et al. 2002), non-small cell lung cancer (An et al 2002; Palmisano et al 2000; Belinsky et al 1998), gastric carcinoma (Lee et al 2002), liver, prostate, bladder and colorectal cancers (Wong et al 1999; Jeronimo et al 2002; Dominguez, 2002; Lecomte et al 2002). Substantial efforts are being made recently for the development of new methods and tools that are capable of sensitive and quantitative DNA methylation analysis, as well as early and accurate diagnosis of cancer. Among such tools are: Fluorescent methylation--specific polymerase chain reaction assay (FMS-PCR; Goessl et al 2000), SNIRF (Mahmood and Weissleder, 2003), indocyanine green-labeling (IGL) for human breast carcinomas (Ntziachristos et al 2000), ConLight-MSP (Rand et al 2002), COBRA (Xlong and Laird, 2002), Methylation-Sensitive *Single Nucleotide* Primer Extension (Ms-SnuPE; Gonzalgo and Jones, 1997), DNA microarray sensitive detection by Metal-Enhanced Fluorescence (MASD/MEF; Lakowicz, 2001; Malicka et al 2003 a, b)), and NIR Fluorescence MicroSpectroscopy (NIRFMS), single cancer cell detection (Baianu et al 2003a).

Specific molecular markers of cancer (Sidransky, 2002) hold the promise to identify those molecular signatures that are *unique* to specific types of cancer, and are essential for the *early accurate diagnosis* and treatment of cancer. Such novel molecular tools and methodologies could be employed to rapidly and accurately identify molecular signatures of cancer and aging-related diseases in mammalian cells in culture in order to determine how specific epigenomic mechanisms involved in the control of cell division and apoptosis operate throughout the cell

cycle. Among the specific epigenomic control mechanisms that one could investigate with such new tools are: CpG-island methylation, p15 (INK4b) and p16 (INK4a) hyper-methylation (in synchronous hepatic carcinoma cells), GSTP1 methylation in non-neoplastic/ synchronous cells, as well as histone-deacetylation and its effects on histone- nucleic acid interactions in stable synchronous cell populations in culture. Both cancer and aging were reported to involve DNA methylation of specific genome regions (van Helden & van Helden, 1989; Ahuja et al 1998). Gene expression profiling and epigenomic testing could be carried out with both ultra-sensitive, novel Human and Mouse microarrays. Powerful spectroscopic and microspectrosopic techniques can be then employed for the analysis and further improvement of such tools for the investigation of Nucleic Acid--Protein interactions.

Table I. **Techniques under Development and Related Applications that are commercially supported*.**

- High-field 2D NMR of protein--protein and protein--nucleic acid interactions

- NIR Chemical Imaging of protein clusters in cells and single cancer cells in tissue

- NIR-FMS

- SNIRF

- MEF and FCS/FCCS/ FRET detection of single molecules amplified-ELISA

- Ms-SnuPE, FMS-PCR

- NASBA

- Lux ^{TM} Fluorogenic Primers*/ RT-PCR*,

- MyArray ^{TM} DNA- Human*, GeneFilters ^{R} Human Regular Arrays**.

- Specific Knock-out or silencing shRNAi's (SuperArrayTM).

**The testing of these new tools can be carried out for example with stable and synchronous mammalian (human HeLa and mouse) cells in culture.

7.   Biotechnology Applications

7.1. Genetic manipulation of Microorganisms for Biotechnology Applications

Genetic manipulation techniques have played an important role in enhancing the performance of microorganisms that are significant in the industrial, pharmaceutical and medical application areas. Such techniques are discussed briefly in this section together with their selected applications. It is important to highlight the fact that they are generic, and also that they can be employed to improve the performance of any cell or microorganism for specific applications. A number of techniques were, and are, successfully employed to obtain such results.

7.1.1. Mutagenesis-based Techniques

Mutagenesis can be defined as the group of techniques or processes causing changes in a DNA sequence that modifies either the expression of genes or the structure of the gene products. Two subcategories of mutagenesis are usually applied in the genetic manipulation of microorganisms: classical mutagenesis and transposon-directed mutagenesis.

Classical Mutagenesis involves the use of chemical mutagens to regulate a process of interest in the target microorganism. The main purpose of this 'classical' approach is to find the mechanism of action of the targetted microorganism.

Transposon-directed Mutagenesis consists in the genetic alteration of a few sites on the target chromosome in order to determine the function of this specific site of the chromosome. By comparison with the previous category transponson- directed mutagenesis it is more specific, and is therefore preferred in specific applications that require higher selectivity.

7.1.2. Gene Transfer

Electroporation involves the production of transient pores in a bacterial cell membrane following the application of high-voltage electric field (DC) pulses of short duration. Such pores allow the introduction of DNA into the cell under certain, favorable conditions. Such methods have been used with various microorganisms and even plasmids that are of significant use in the industry.

Another technique that is employed for the transference of genes involves Shuttle Vectors. *Shuttle vectors* are DNA constructs that are able to replicate and deliver DNA to widely divergent types of bacteria (Blaschek 1996). DNA transference is obtained after the use of a microorganism whose mechanisms of action have been thoroughly studied, as for example, is the case of various *E. coli* strains.

7.1.3. DNA Analysis

DNA analysis involves the study of a particular region of DNA by either physical or chemical techniques. It is usually carried out in order to obtain insights into the genetic organization, the mechanisms of gene expression, or to obtain information that may be useful for the construction of shuttle vectors (Blaschek, 1996).

7.1.4. *Suggested Conceptual and Computational Approaches to Genomic and Interactomic Studies of Microorgansims and Higher Organisms*

Quantitative, biological systems approaches, in conjunction with both algebraic and topological models, are capable of simulating a wide range of non-linear behaviors exhibited by the highly-adaptive, coupled genetic and protein networks that are regulating the stress responses in unicellular systems, such as selected microorganisms. Very large data sets were recently gathered through extensive genomic and proteomic microarrays and functional microanalyses of a rapidly increasing number of microorganisms. Several, rather intriguing types of coupled genetic and protein networks are capable of a very wide range of stress responses in extremophilic microorganisms that strive in quite challenging habitats. For example, it would be very interesting to carry out a comparative modeling of major differences between stress response, or challenge, mechanisms in unusual, extremophilic microorganisms and several, selected non-extremophilic, Gram-positive bacteria. The bacterial strains selected should have already available, preferably complete genomic, as well as extensive proteomic, databases that are also of considerable practical importance in several emerging biotechnology applications.

A *complex system analysis* may allow one to perform realistic simulations through fast parallel computations, as well as multi-stability and careful sensitivity analyses. Suitable representations of such coupled genetic and protein networks in terms of nonlinear bio-control models will involve stresses and perturbations related to hidden Markov bioprocesses in nonpathogenic microorganisms. Such quantitative modeling is now being made possible by the extensive data collections in existing biotechnology databases. The related measurements, model refinements and validation tasks will require very accurate data which can be obtained in single cells of selected microorganism strains. Novel ultra-sensitive, high throughput screening techniques would also be required in order to acquire reliable proteomic data that can provide critical constraints for computations of meaningful biodynamics simulations.

A number of theroretical predictions have anticipated by as many as 30 years experimental findings in molecular biology and oncogenetic research. Among such predictions are: the existence, as well as function / structural domains, of telomeres and telomerases together with their potential roles in cancer (Baianu, 1969), specific changes in somatic (or stem) cells' dynamic programming that are causing neoplastic transformations and also altered epigenetics in cancer cells (Baianu, 1969, 1971), the linking of gene functions and complex non-linear dynamics exhibited by genetic networks of human cells (Baianu, 1971, 1977, 1987), and the existence of a *homology sequence* of conserved ('linked') gene clusters (Baianu, 1971) and 'essential' proteins (Baianu and Marinescu, 1968; Baianu, 1969, 1987) throughout biological evolution. More recent predictions that are yet to be checked out through experiments concern specific changes in the functional links of the cell cycling control 'module' with several key signaling pathways (Baianu, 2004a,b,c; Prisecaru and Baianu, 2004; Baianu, 2006) that frequently occur in human cancer cells.

8. Conclusions

Novel translational oncogenomics research is rapidly expanding with a view to the application of new technologies, findings and computational models in both pharmaceutical and clinical areas. Sample analyses in recent clinical studies have shown that gene expression data can be employed to distinguish between tumor types as well as to predict outcomes. Important, potential applications of such results are *individualized* human cancer therapy (Pharmacogenomics) and 'personalized medicine'. There is clearly a need for individualized cancer therapy strategies based on high-throughput microarray information recorded for isolated tumor cell lines from stage I through stage III cancer patients. Studies of Differential Gene Expression in human cancer cell lines are clearly required for developing new strategies for efficient cancer therapies for patients whose tumors have developed resistance to existing therapies. Such gene profiling expression, proteomic, interactomic and tissue array data is essential for improving the survival rate of stage III cancer patients undergoing clinical trials with novel signaling pathway inhibitors/blocker medicines, such as those discussed in some detail in Section 5. Several technologies aimed at future applications in oncogenesis are currently under development both in the direction of improved detection sensitivity and increased time resolution of cellular events, with the limits of single molecule detection and picosecond time resolution already being reached (Sections 2.6, 2.7 and 6.2). The urgency for funding and carrying out the complete mapping of a human cancer interactome with the help of such novel, high-efficiency / low-cost and ultra-sensitive techniques is pointed out for the first time in the context of recent findings by translational oncogenomics and human cancer interactome predictions.

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